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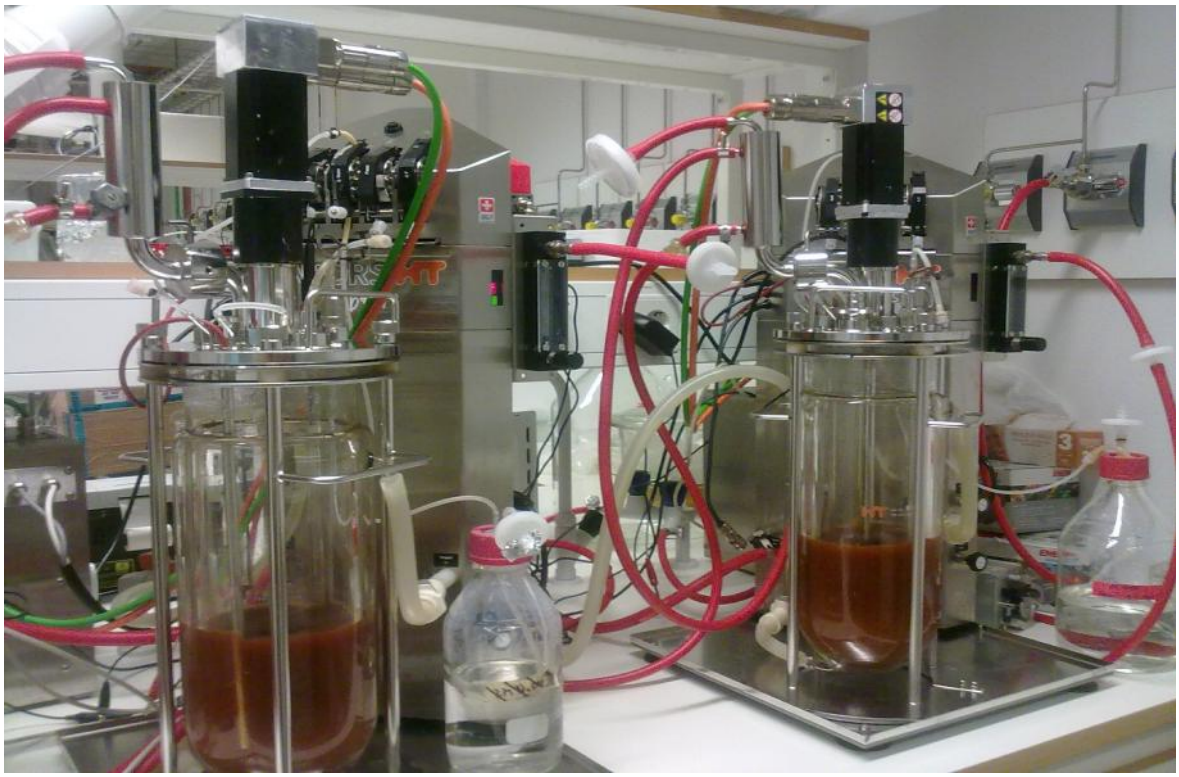

STUDIES USING XYLOSE FERMENTING YEAST FOR LIGNOCELLULOSIC FERMENTATION.

Master of Science Thesis

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"Studies using xylose fermenting yeast for lignocellulosic fermentation"

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Preface

This project was performed for the partial fulfilment of Master of Science in Biotechnology at Chalmers University of Technology. The work was carried out from September 2011 until June 2012 at the Industrial Biotechnology lab, Department of Chemical and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden.

Abstract

Lignocellulosic hydrolysates such as spruce and *Arundo donax* were used in Separate Hydrolysis and Fermentation (SHF) process to analyse the fermentative performance of each recombinant *Saccharomyces cerevisiae* strain: GS1.11-26 and VTT C-10883, engineered for xylose utilization. In shake flask experiments, GS1.11-26, an industrial strain showed better fermentative performance and thereby it completely consumed xylose present in spruce compared to the other laboratory strain, VTT C-10883. The complete consumption of xylose by the industrial strain was not observed in *Arundo*. Furthermore, analysis of both strains in bioreactors showed that GS1.11-26 has higher ethanol production rate and ethanol yield on consumed sugars in both spruce (0.42 g/g) and *Arundo* (0.38 g/g).

In spruce material, in addition to sugars, inhibitors such as furaldehydes [furfural and hydroxymethyl furfural (5-HMF)] are generally present in high amounts. The compounds are generated during the pretreatment and saccharification process of lignocellulosic biomass to ethanol. Most yeast strains has limited tolerance ability towards these inhibitors and the current research focuses on the development of stress tolerant strains to efficiently convert lignocellulose derived raw material into fuels and chemicals. In the second part of the project, pulse addition experiments were carried out using HMF and furfural to analyse the stress response of VTT C-10883 towards inhibitors. The addition of 3.9 g/L HMF and 1.2 g/L furfural to the exponential growth phase of VTT C-10883 resulted in decreased specific growth rate [0.07 (±0.006) h^{-1}] compared with the control [0.28 (±0.02) h^{-1}]. The amount of ethanol produced decreased for 2-3 hours after pulse addition, however, at the end of fermentation, approximately 20 g/L ethanol was produced in both pulse and control samples. Also, an increase in the average glycerol yield was observed in high concentration inhibitor pulsed samples. The average yield of acetate increased and xylitol, a major by-product produced during anaerobic xylose metabolism, was found to significantly decrease in the presence of inhibitors. The results of this study showed that the inhibitors furfural and 5-HMF were completely metabolized by VTT C-10883 strain. In the future, more metabolic studies performed using this strain could result in strategies to improve tolerance towards inhibitors.

Content

1 INTRODUCTION

In recent years, there has been increasing interest towards sustainable energy resource such as biofuels due to the rise in petroleum prices and environmental problems related to green house gas (GHG) emissions (Prasad et al, 2007). Since ethanol is an oxygenated fuel, it can be easily blended with gasoline and can be widely used for transportation purpose across the globe. The use of biofuels will significantly lower the emission of exhaust gases thereby resulting (Demirbas, 2007) in a clean and eco-friendly environment.

The first generation bioethanol is produced mainly from different crops such as corn, wheat, sugarcane, rice and barley. The usage of these crops for ethanol production led to its shortage for food (Pimentel et al, 2008) and currently, renewable biomass such as lignocellulosic material has become an attractive low cost material for ethanol production, so called second generation bioethanol (Bothast and Saha, 1997). Lignocellulose consists of three main components: cellulose, hemicellulose (both referred as carbohydrate polymers) and lignin (aromatic polymer). Cellulose is a glucose polysaccharide while hemicelluloses are polysaccharides with different sugars such as hexoses (glucose, mannose, and galactose) and pentoses (xylan, arabinose). Lignin is covalently bonded to cellulose and hemicellulose and thereby provides with compressive strength and stiffness to the plant tissue and cell wall (Del Rio et al, 2007). Due to close association of lignin with carbohydrate polymers, lignocellulosic material must undergo pretreatment to break down lignin and also for the sugars to be available during fermentation. After pretreatment, lignocellulosic material undergoes acid/enzymatic hydrolysis to release sugar monomers from the carbohydrate polymers and lastly, the sugars present in the hydrolysate material are fermented to produce ethanol using baker's yeast, *Saccharomyces cerevisiae*. Having the ability to ferment glucose, these wild type strains cannot naturally ferment xylose and over the past decades research has been carried out to develop recombinant *S. cerevisiae* to ferment xylose as well in order to achieve higher ethanol production.

During the conversion of renewable biomass to ethanol, the use of high temperature and pressure in various pretreatment and hydrolysis processes generate different toxic compounds in addition to sugars. These toxic compounds/inhibitors include weak acids, furaldehydes and phenolic compounds (Palmqvist et al, 1999). Higher amounts of furaldehydes generated during the dilute acid hydrolysis process are more toxic. It includes furfural and hydroxymethylfurfural (5-HMF) which are generated by the dehydration of pentoses (Dunlop, 1948) and hexoses (Ulbricht et al, 1984).

During batch cultivations of *S. cerevisiae*, furfural acts as a strong inhibitor (Boyer et al, 1992) inhibiting the in vitro activity of glycolytic enzymes (Palmqvist et al, 1999 and Modig et al, 2002), and thereby results in decrease of cell multiplication, CO_2 production and total viable cell number. In pulse experiments performed under anaerobic conditions, the specific growth rate of

strain was reduced when different concentrations of furfural (Taherzadeh et al, 1999) and 5-HMF (Taherzadeh et al, 2000) were pulsed. Increase in acetaldehyde accumulation and decrease in specific glucose consumption and ethanol production rate were observed. These compounds also break down DNA (Modig et al, 2002) and cause lag phase during fermentation (Taherzadeh et al, 1999). The fermentation process can be enhanced in several ways. Firstly, the pretreatment and hydrolysis process can be optimized to minimize inhibitor formation. Secondly, detoxification methods can be developed to remove inhibitors prior to fermentation, and, lastly, efficient yeast strains tolerant to inhibitor complexes must be developed to convert the inhibitors present in the lignocellulosic material.

Here in the first part of project, xylose fermenting ability of two recombinant *S. cerevisiae* strains were analysed (Fig: 1) and in the second part (Fig: 2), tolerance ability of a recombinant *S. cerevisiae* strain towards inhibitors were also analyzed.

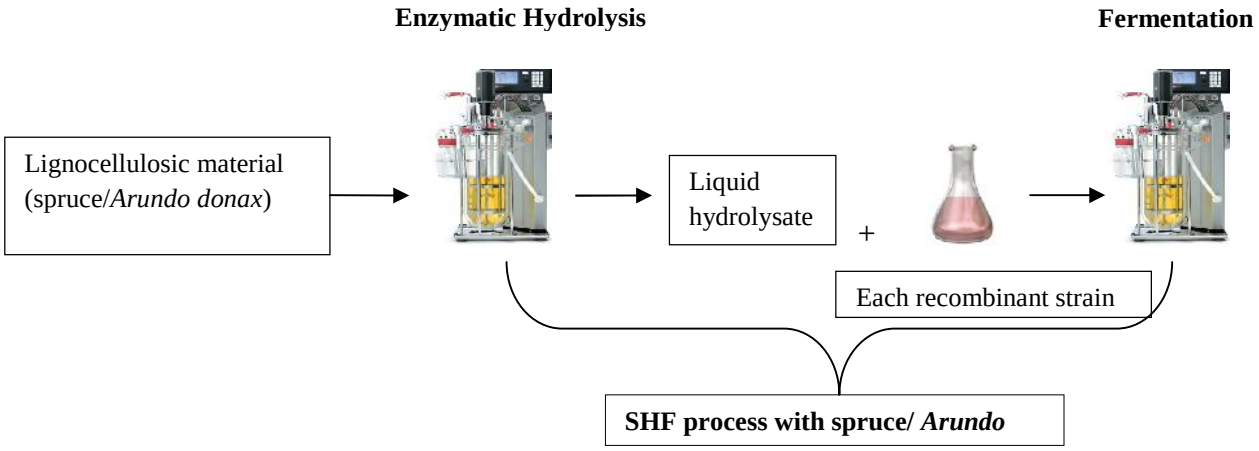


Fig: 1 Schematic representation of Separate Hydrolysis and Fermentation (SHF) process performed with two different lignocellulosic materials: spruce and *Arundo donax* using two different recombinant *S. cerevisiae* strains, GS1.11-26 and VTT C-10883.

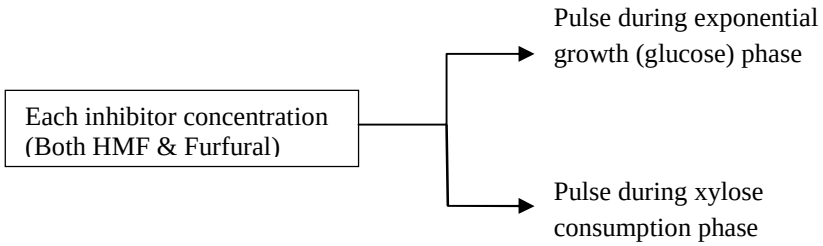


Fig: 2 Schematic representation of Pulse experiments with different concentration of both HMF and Furfural pulsed during the exponential growth phase and xylose phase of recombinant *S. cerevisiae* strain VTT C-10883.

In the first part of this project, (Fig: 1) xylose fermenting ability of two recombinant *S. cerevisiae* strains, GS1.11-26 and VTT C-10883 were analyzed using two different lignocellulosic materials such as spruce and *Arundo donax*. Separate Hydrolysis and Fermentation (SHF) process was carried out to analyze the growth and fermentative performance of each strain towards these two hydrolysates.

In Spruce, furaldehyde compounds (5-HMF & Furfural) present in higher amounts are toxic and based on literature studies, the presence of these compounds in the media/hydrolysate material affect the growth and fermentation performance of yeast strain. So in the second part of this project (Fig: 2), physiological studies were performed using recombinant *S. cerevisiae* strain, VTT C-10883 to analyze its tolerance ability towards these compounds. Pulse addition experiments were performed preliminarily in shake flasks and later in bioreactors. In shake flasks, three different increasing concentrations of both HMF and furfural were pulsed during two different growth phases [exponential growth phase (glucose phase) and xylose phase] of the strain. The inhibitor concentration which showed maximum difference in specific growth rate, ethanol and xylitol yield compared with the control was chosen for pulse experiments in bioreactors.

2 AIM

The project involves two different experimental parts: the first part (SHF PROCESS) aims to compare the fermentation performance of two strains: GS1.11-26 and VTT C-10883 (recombinant *Saccharomyces cerevisiae* strains engineered for xylose fermentation) by using two different industrial hydrolysates such as spruce and *Arundo donax* for bioethanol production.

The second part (PULSE EXPERIMENTS) aims to study, analyse and understand the response of VTT C-10883 towards the presence of inhibitors, mainly furaldehydes such as furfural and 5-HMF in a defined media. The specific effects of furaldehydes towards cells can be understood by analysing the extracellular metabolites and parameters related to the energy metabolism [mainly adenonucleotides-AXP (ATP/ADP/AMP)].

3 BACKGROUND

3.1 Biofuels

Biofuel usually refers to solid (bio-char), liquid (ethanol, biodiesel and vegetable oil) and gaseous (biogas, biosyngas and biohydrogen) fuels derived from biomass. The organic/biological materials obtained from forest, agricultural residues, municipal solid wastes, and other wastes from food and agro industries are usually referred as biomass. Biofuels produced from biomass have the ability to reduce CO_2 emission as the plants use CO_2 to grow (Osamu et al, 1989). It is important that the amount of carbon molecules released in the environment should be balanced, a concept of closed carbon cycle (Fig: 3); the imbalance of carbon by the use of fossil fuels resulted in increase of CO_2 concentration in the environment (Bigg et al, 2003). The increase in oil consumption and emission levels with combined fossil fuel depletion led to an increase in demand for biofuel, a sustainable and renewable energy source (Prasad et al, 2007). It is used as an alternative choice of energy consumption due to its sustainability and acceptable quality of emission gases (Demirbas, 2009).

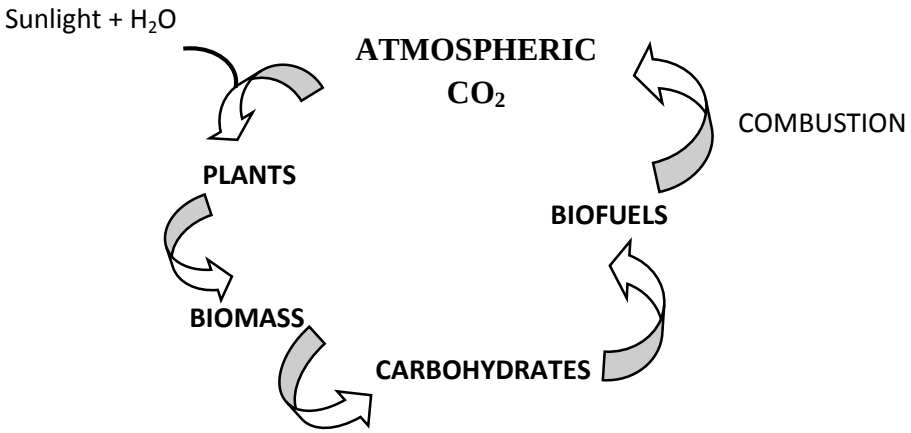


Fig: 3 Closed carbon cycle (modified & redrawn from (van Maris et al, 2006))

3.2 Bioethanol

Bioethanol is commonly used for transportation sector (Balat and Balat, 2009). The feedstock for bioethanol production might vary and can be starch based materials (wheat, barley, rice, maize, corn and potatoes), sucrose containing feedstock (sugarcane, sugar beet, sweet sorghum and fruits) and lignocellulosic feedstock (straw, wood and grasses). It is produced by the action of microorganisms on the fermentable sugars present in the raw material. Ethanol contains 35% by weight of oxygen and it can be used directly or blended with gasoline for transportation means. The use of partially oxygenated fuel in automobiles significantly reduces vehicle exhaust emission (Demirbas, 2005) and greenhouse gas emission (Balat, 2009).

3.3 Bioethanol production

3.3.1 Classification of bioethanol production

The commercial use of ethanol provides sustainable environment to mankind and suitable process improvements are being carried out in large scale to increase ethanol production. On the basis of available raw material sources, bioethanol production is classified as first and second generation bioethanol.

3.3.1.1 First generation bioethanol

The first generation bioethanol is produced from starch and sucrose containing feedstock. Based on the geographic location, the feedstock availability varies for bioethanol production. The most widely used crops for first generation bioethanol production include sugarcane, corn, wheat, sugar beet and barley. At present, first generation bioethanol is commercially produced in many countries and the process involves separation of sugars from the raw material followed by the fermentation of sugars. Distillation and dehydration are lastly carried out to obtain a desired concentration of ethanol. The increase in use of food crops for bioethanol production led to its shortage and in turn increased the price of food commodities (Pimentel et al, 2008). The commercial production of ethanol in the upcoming years seems to be limited because of the competition in water and land availability for crop production (Searchinger, 2008). These limitations can be overcome by the use of non-edible biomass sources rather than food crops in ethanol production. The good infrastructure and well implemented process employed in the first generation bioethanol production facilitate necessary support and background for the development of second generation bioethanol.

3.3.1.2 Second generation bioethanol

Second generation bioethanol is obtained from non-food renewable feed-stocks known as lignocellulosic biomass. It is most abundantly present on earth and it constitutes the majority of plant waste organic matter such as straw, cornhusk, wheat, sugarcane bagasse, corn stover, wood chips, saw dust and organic fraction of municipal solid waste. In general, the process of ethanol production from lignocellulosic biomass includes the following key steps: pretreatment of lignocelluloses; chemical or enzymatic hydrolysis of cellulose and hemicellulose fraction; fermentation of sugars, which include SHF (Separate Hydrolysis and Fermentation) or SSF (Simultaneous Saccharification and Fermentation) or CBP (Consolidated Bioprocessing), recovery and distillation (Balat, 2009). The use of lignocellulosic biomass for ethanol production limits food competition (Searchinger, 2008) and liberates lower amounts of greenhouse gases (GHG) (Demirbas, 2005).

3.4 Lignocellulose composition

Lignocellulose constitutes a major portion of plant dry matter and has three major components such as cellulose (40-45%), hemicellulose (20-30%) and lignin (15-25%). Carbohydrate polymers such as cellulose and hemicellulose contain different sugar monomers whereas lignin, an aromatic polymer is synthesized from phenylpropanoid precursors (Fig: 4). The carbohydrate and aromatic polymers arrange to form structures called microfibrils, which further organize to form structures called macrofibrils, which mediate cellular stability in the plant cell wall (Rubin, 2008). The composition of various lignocellulosic components vary between plant species (Prasad et al, 2007) depending upon growth conditions (Goel et al, 1996), and part of plant (Barl et al., 1995). In general, the presence of high cellulose and lignin content in wood provides rigidity and high hemicellulose content favours flexibility to straw.

3.4.1 Cellulose

Cellulose, the structural component of primary plant cell wall constitutes the major portion of lignocellulosic material. It is a linear homopolysaccharide chain containing many D-glucose subunits linked together by β-1, 4 glycosidic linkages and hydrogen bonds are present between the polysaccharide chains. The orientation of glycosidic linkages and hydrogen bonds provide rigid support to the polymer and makes crystalline cellulose difficult to break during hydrolysis (Rubin, 2008). In the cell wall of biomass, cellulose is strongly protected by hemicellulose and lignin, which makes it resistant to microbial and enzymatic degradation (Himmel, 2007).

3.4.2 Hemicellulose

Hemicellulose, the second most abundant component present in lignocellulose is a low molecular weight heteropolysaccharide chain containing two different sugar monomers such as hexoses (glucose, mannose and galactose) and pentoses (arabinose and xylose). The sugars are linked together by β-1, 4 glycosidic linkages and β-1, 3 glycosidic linkages. The highly branched structure and amorphous nature of hemicellulose makes it readily susceptible to hydrolysis compared to the crystalline structure of cellulose. The dominant sugar monomers of softwood and hardwood include mannose and xylose, respectively (Taherzadeh et al, 2008).

3.4.3 Lignin

Lignin, a complex aromatic heteropolymer (Kirk and Farrell, 1987) is synthesized by the polymerization of three different phenyl propane units, namely p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. The three different phenyl propane units are linked together by non-hydrolysable linkages to form a complex matrix (Demirbas, 2008). It is covalently bonded to both cellulose and hemicellulose and makes lignocelluloses resistant to hydrolysis (Taherzadeh et al, 2008). Higher amounts of lignin are present in softwoods compared to hardwoods (Demirbas, 2008).

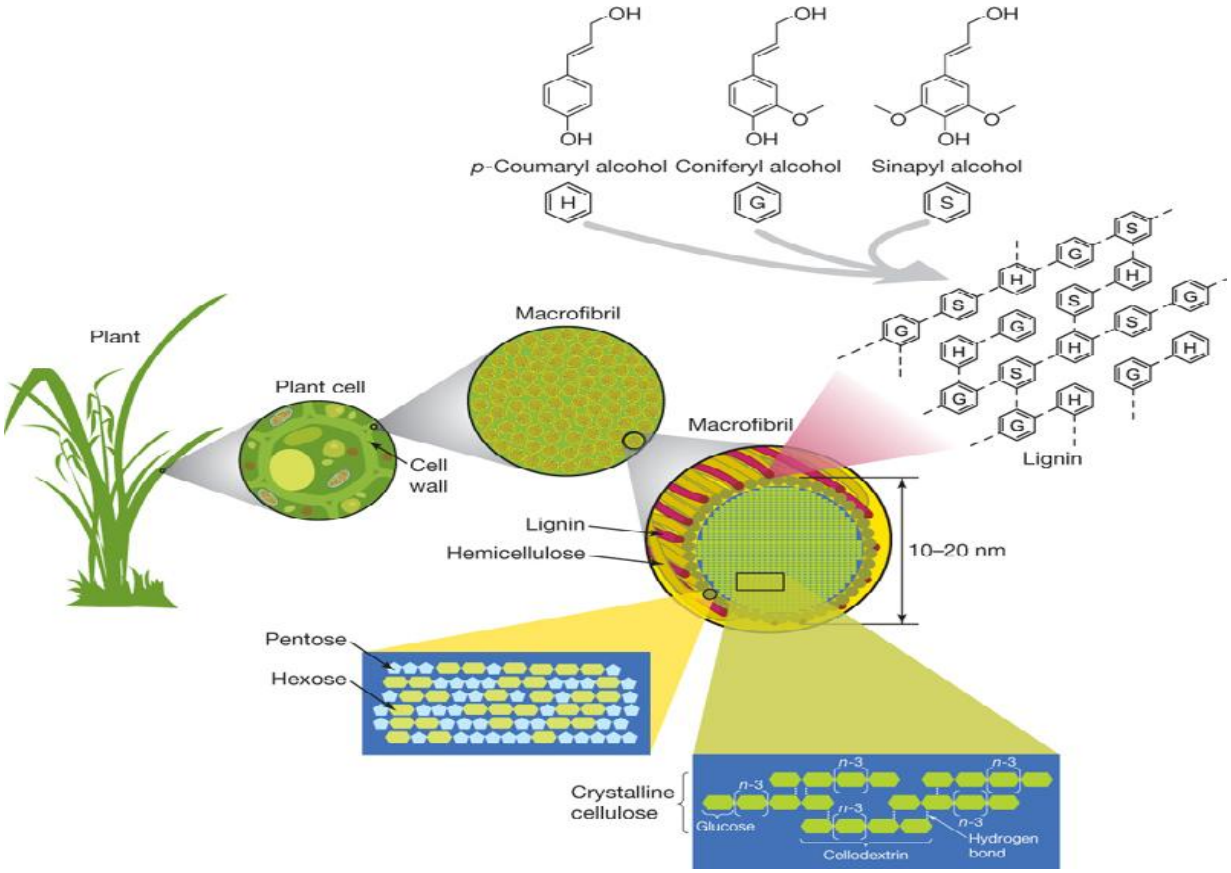


Fig: 4 Structure of lignocellulose material **(adopted from Rubin, 2008)**

3.4.4 Lignocellulosic materials

In this project, two different pretreated lignocellulosic materials such as spruce and *Arundo donax* were used. Spruce is a large coniferous evergreen tree (20-60 metres high) usually found in the northern regions of earth. More amounts of glucose and less xylose are present in cellulose and hemicellulose polymers, respectively. *Arundo donax* is a perennial grass (3-6 m high) variety found in the mediterranean region. It has lower amounts of glucose and higher amounts of xylose compared with spruce.

3.5 Process steps involved in the production of ethanol from lignocellulose

Lignocellulosic material needs to be chipped and grinded to produce lignocellulosic biomass, which undergoes different process steps (Fig: 5) to produce desired concentration of ethanol.

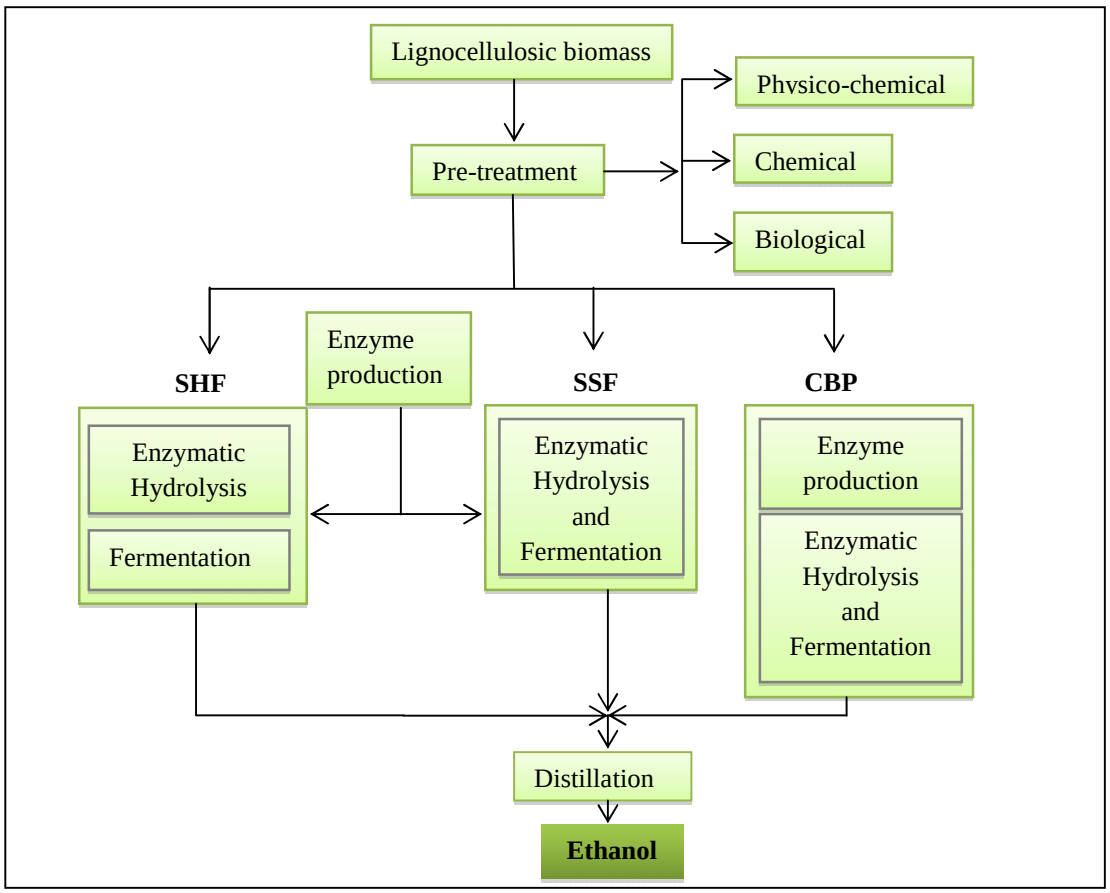


Fig: 5 Schematic representation of process steps involved in the production of ethanol from lignocellulosic biomass. * SHF- Separate Hydrolysis and Fermentation, SSF- Simultaneous Saccharification and Fermentation, CBP- Consolidated Bioprocessing.

3.5.1 Pre-treatment of lignocellulose

Pre-treatment of lignocellulose is a prerequisite step carried out to increase the surface area of the feed stock, remove barriers made by lignin and hemicellulose, to increase porosity of cellulose and to release the sugars from cellulose by enzymatic hydrolysis (Zhu et al, 2008). Inefficient pre-treatment processes might result in non-hydrolysable residue formation and in more severe cases it also results in the production of toxic inhibitory compounds that greatly inhibit the microbial growth and affect the fermentation process (Kodali et al, 2006). The pre-treatment can be classified into physiochemical, chemical and biological processes.

3.5.1.1 Physicochemical pretreatment

3.5.1.1.1 Steam explosion

Steam explosion (auto hydrolysis) has been the most commercially used pre-treatment method for lignocelluloses (McMillan, 1994). In the process described by Saddler et al, 1993, chipped

biomass was exposed to high-pressure saturated steam of 0.69-4.83 MPa (corresponding temperature 160-260°C) for several seconds and up to 10 minutes. In later steps, the high-pressure saturated steam was reduced to make the lignocellulosic material to undergo explosive decompression. High temperatures might provide improved cellulose digestibility, increased removal of hemicelluloses, lignin transformation but cause sugar degradation. The addition of acid catalyst such as H_2SO_4 or SO_2 in steam explosion pretreatment has been recognized to decrease the production of sugar degradation compounds, improve digestibility of cellulose and improve the hydrolysis of hemicelluloses (Sun and Cheng, 2002). In this technique, the use of SO_2 compared to H_2SO_4 results in more digestible substrate with high fermentability and also promotes high xylose recovery from hemicelluloses (Zheng et al, 2009). The steam explosion pre-treatment is considered to be the most cost effective method for hardwoods and agricultural residues (Prasad et al, 2007).

3.5.1.1.2 Ammonia Fiber Explosion (AFEX)

In AFEX pre-treatment, dry lignocellulosic materials are impregnated with liquid ammonia and heated to a temperature of 50-90°C under pressure (10-20atm) for a few minutes and later the pressure is released. The change in pressure causes disruption of fiber structure, thereby causing structural alteration of cellulose and lignin (Holtzapple et al, 1991). The alteration increases the accessible surface area of cellulose and results in improved enzymatic hydrolysis. The steam and AFEX pre-treatment methods were compared for the enzymatic hydrolysis of aspen wood, wheat, straw and alfalfa by Mes-Hartree et al, 1988 and identified that this pre-treatment did not considerably solubilize hemicelluloses compared to steam explosion technique. Herbaceous crops and grasses with low lignin content are efficiently pre-treated in this process compared to high lignin content materials e.g., softwood (Vlasenko et al, 1997; Holtzapple et al, 1991). The advantages include low inhibitor formation (Mes-Hartree et al, 1988) and no requirement of small particle size for efficacy (Holtzapple, et al, 1991).

3.5.1.2 Chemical pre-treatment

3.5.1.2.1 Acid pretreatment

Dilute acid pretreatment process has been carried out in two different stages to avoid the formation of sugar degradation products and also to achieve high yield of sugars from lignocelluloses. The first stage utilizes low temperature (less than 160°C) and operates in batch process for high solid loading (10-40% (w/w) (Cahela et al, 1983; Esteghlalian et al, 1997). The second stage is carried out at higher temperatures (greater than 160° C) and uses continuous flow process for low solid loading (5-10%) (w/w) (Brennan et al, 1986; Converse et al, 1989). The low temperature stage significantly removes five carbon sugars and the second high temperature stage significantly removes six carbon sugars from hemicellulose and cellulose, respectively(Hamelinck et al, 2005). The two-stage recovery process involves high cost and significantly improves cellulose hydrolysis.

3.5.1.2.2 Alkaline pretreatment

Alkaline pre-treatment uses chemicals such as sodium, potassium, calcium and ammonium hydroxide and utilizes lower temperature compared to other pretreatment technologies. This process aims to remove lignin and various uronic acid substitutions in hemicellulose which lowers the enzyme accessibility to carbohydrate polymers (Silverstein et al, 2008). NaOH pretreatment is the most commonly studied alkaline pretreatment process (Kumar et al, 2009) and it causes swelling of lignocellulosic materials which allow the separation of structural linkages between lignin and carbohydrate polymers, decreases cellulose crystallinity and cause lignin disruption which might lead to an increase in internal surface area (Fan et al, 1987). The digestibility of dilute NaOH pre-treated hardwoods are found to increase from 14% to 55% with suitable decrease in lignin content from 24-55% to 20% compared to softwoods with high lignin content (Millet et al, 1976).

3.5.1.3 Biological pre-treatment

Biological pre-treatment employs microorganisms such as white-, soft- and brown-rot fungi to degrade lignin and solubilize hemicellulose in lignocellulosic materials. In response to carbon and nitrogen limitation during secondary metabolism, white-rot fungus *P. chrysosporium* produce lignin degrading enzymes, lignin peroxidases and manganese peroxidases to remove lignin present in the materials (Boominathan and Reddy, 1992). The biological pre-treatment of lignocellulosic materials has been effectively carried out by white-rot fungi as it was able to reduce lignocellulose recalcitrance to enzymatic hydrolysis (Itoh et al, 2003). White-rot and soft-rot fungi attack cellulose and lignin polymers while brown-rot fungi attack only cellulose. Advantages of biological pre-treatment include low energy requirement and mild environmental conditions. The major drawback of this pre-treatment method is very low rate of hydrolysis (Sun and Cheng, 2002).

3.5.2 Hydrolysis

After pretreatment, lignocellulosic material consists only of cellulose and lignin as the hemicelluloses are completely solubilised during the pretreatment process. Later, in order for the sugars to release from cellulose structure, the material undergoes hydrolysis which may be acid and enzymatic. During acid hydrolysis, sugar degradation compounds are formed which can be prevented by using enzymes in the hydrolysis process to convert cellulose to glucose at an optimum temperature.

Enzymes are produced by different microorganisms including bacteria or fungi. These are specific in nature and mainly cellulases are being produced in large quantities. In hydrolysis, the use of carbohydrate degrading enzymes (cellulases and hemicellulases) for the release of fermentable sugars from the pre-treated lignocellulosic material for ethanol production is known as enzymatic hydrolysis (Keshwani and Cheng, 2009).The most widely studied cellulolytic bacteria and fungi for cellulase production include *Cellulomonas fimi, Thermomonospora fusca* and *Trichoderma reesei* (*Hypocrea jecorina*). EC system, International Union of Biochemistry

and Molecular Biology classified cellulytic enzymes into three different classes based on their substrate specificity and molecular mechanism(Henrissat et al, 1998) Table: 1.

Table: 1 Representation of three different classes of enzymes

S.No	Enzyme classes	Activity
1	exo-1,4-β-D-glucanases or cellobiohydrolases (CBH) (EC 3.2.1.91)	Cleaves cellobiose units from ends of cellulose chains
2	endo-1,4-β-D-glucanases (EG) (EC 3.2.1.4)	Hydrolyse internal β-1,4-glucosidic bonds randomly in the cellulose chain
3	1,4-β-D-glucosidases (EC 3.2.1.21)	Hydrolyse cellobiose to glucose; cleaves glucose units from cello oligosaccharides

3.5.3 Fermentation

After hydrolysis, the fermentation process takes place where the released sugars from the hydrolysis process are consumed by yeast, *S. cerevisiae* to produce ethanol. Enzymatic hydrolysis and fermentation process after pretreatment can either be performed separately or simultaneously, which are referred as Separate Hydrolysis and Fermentation **(SHF)** or Simultaneous Saccharification and Fermentation **(SSF)** processes. In SHF process, enzymatic hydrolysis and fermentation process takes place in two different vessels separately at 45-50°C and 30°C, respectively. Recycling of yeast cells is possible here but the sugars released during the hydrolysis process might inhibit the enzymes. In SSF, both these hydrolysis and fermentation process are carried out as one process step and cell recycling is not possible here. The sugars released during the hydrolysis process do not inhibit the enzymes as they are consumed immediately by *S. cerevisiae* to produce ethanol.

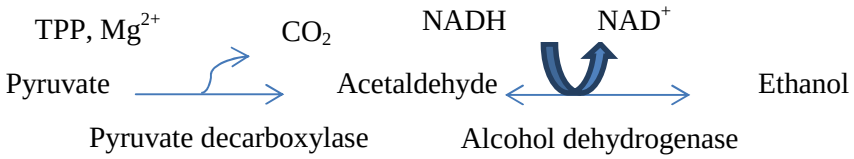
3.5.3.1 *Saccharomyces cerevisiae*- fermenting organism for bioethanol production

The eukaryotic microorganism *S. cerevisiae* commonly referred as bakers' or brewers' yeast is used as a catalyst for the production of food and alcoholic beverages. It is a traditional industrial microorganism used to ferment the sugars present in the raw material for the production of ethanol. It has an efficient anaerobic sugar metabolism and its robustness, inhibitor tolerance, elevated osmotic pressure tolerance and high ethanol productivity (Hahn-Hägerdal and Pamment, 2004) make *S. cerevisiae* one of the best suitable microorganisms for fermentation under anaerobic conditions.

It ferments six carbon sugars such as glucose through the glycolysis pathway and it is naturally not able to ferment pentose sugars such as xylose. Glycolysis is the universal central pathway for glucose catabolism and it is divided into two phases: "preparatory phase" and "pay off" phase. 2 molecules of pyruvate are synthesized from one glucose molecule and the reactions are catalysed by different enzymes. The released free energy from glucose is conserved in the form of ATP

(adenosine triphosphate) and NADH (Nicotinamide adenine dinucleotide). 2 molecules of ATP are consumed in the preparatory phase and in turn 2 molecules of ATP are generated per mole of pyruvate produced during the payoff phase through substrate level phosphorylation. The first molecule of ATP is generated with the help of the enzyme phosphoglycerate kinase which transfers a phosphoryl group from the carboxyl group, 1, 3-bisphosphoglycerate to ADP (adenosine diphosphate) and a second molecule of ATP is generated with the help of enzyme pyruvate kinase which transfers a phosphoryl group from phosphoenolpyruvate to ADP. Mg^{2+} or K^{+} is also required to catalyse the ATP generation reaction. The reduced coenzyme, NADH is generated during payoff phase with the help of enzyme glyceraldehyde dehydrogenase which transfers a hydride ion from the aldehyde group of substrate, glyceraldehyde 3-phosphate to the Nicotinamide ring of NAD^{+}, which is regenerated under anaerobic conditions. (Lehninger Principles of Biochemistry Book)

Under anaerobic conditions, pyruvate is either converted to ethanol and CO_2 by yeast or converted to lactate by lactic acid bacteria. Fermentation is the general term used for the reactions under anaerobic conditions, which uses energy stored as ATP but do not consume oxygen. Equation: 1represents the two step conversion of pyruvate to ethanol by *S. cerevisiae.*



Equation: 1 Two-step conversion of pyruvate to ethanol under anaerobic conditions catalysed by pyruvate decarboxylase and alcohol dehydrogenase.

The conversion of pyruvate to acetaldehyde liberates CO_2 and the irreversible reaction is catalysed by pyruvate decarboxylase which requires Mg^{2+} and coenzyme, thiamine pyrophosphate. The second step, catalysed by alcohol dehydrogenase involves the conversion of acetaldehyde to ethanol with the help of reducing power NADH. (Lehninger Principles of biochemistry book, 4th edition) Thus the overall equation (Equation: 2) under anaerobic condition is given by

$$\text{Glucose} + 2\ \text{ADP} + 2\ \text{Pi} \longrightarrow 2\ \text{ethanol} + 2\ CO_2 + 2\ \text{ATP} + 2\ H_2O$$

Equation: 2 Overall formation of ethanol from glucose under anaerobic conditions.

3.5.3.2 Metabolic pathway for xylose utilization

The naturally occurring *S. cerevisiae* strains cannot utilize pentose sugars. Kuhn et al, 1995 and Richard et al, 1999 reported the presence of xylose utilization genes in *S. cerevisiae*. The low expression levels of the endogenous xylose genes in *S. cerevisiae* do not support growth on xylose. Efficient pentose fermentation is necessary to attain economically feasible ethanol production.

Bacteria, fungi and yeast have been identified for efficient xylose fermentation (Jeffries et al, 2004 and Dien et al, 2003). *Candida shehatae, Sheffersomyces stipitis* (Prior and Kilian, 1989) and *Pachysolen tannophilus* (Schneider and Wang, 1981) have the ability to ferment pentoses mainly xylose to ethanol. Gunsalus et al, 1955 first described the pathway that links xylose metabolism to non-oxidative phase of pentose phosphate pathway by the conversion of xylose to xylulose-5-phosphate (

Fig: 6). The genes *XYL1* and *XYL2* coding for xylose reductase (Takuma et al, 1991) and xylitol dehydrogenase (Kötter et al, 1990) were isolated from *Sheffersomyces stipitis* and cloned in *S. cerevisiae* strain for xylose fermentation. The two oxidoreductases: xylose reductase (XR) and xylitol dehydrogenase (XDH) catalyses the conversion of xylose to xylitol and xylitol to xylulose in the presence of NAD(P)H and NAD^+, respectively. Xylitol was the main product formed (Walfridsson et al, 1997). In *S.cerevisiae*, overexpression of gene *XKS1* coding for xylulokinase (XK) (Ho et al, 1998) results in efficient xylose fermentation to ethanol. NAD(P)H dependent XR and NAD^+ dependent XDH results in redox imbalance as NAD^+ cannot be regenerated. In order to maintain redox balance and ferment xylose to ethanol under anaerobic conditions, electron acceptors such as furfural (Wahlbom and Hahn-Hägerdal, 2002) or acetoin (Bruinenberg et al, 1983a) is required to reoxidize NADH. In the absence to reoxidize NADH, the conversion of xylose to xylulose is limited and results in xylitol accumulation and excretion (Bruinenberg et al, 1983a).

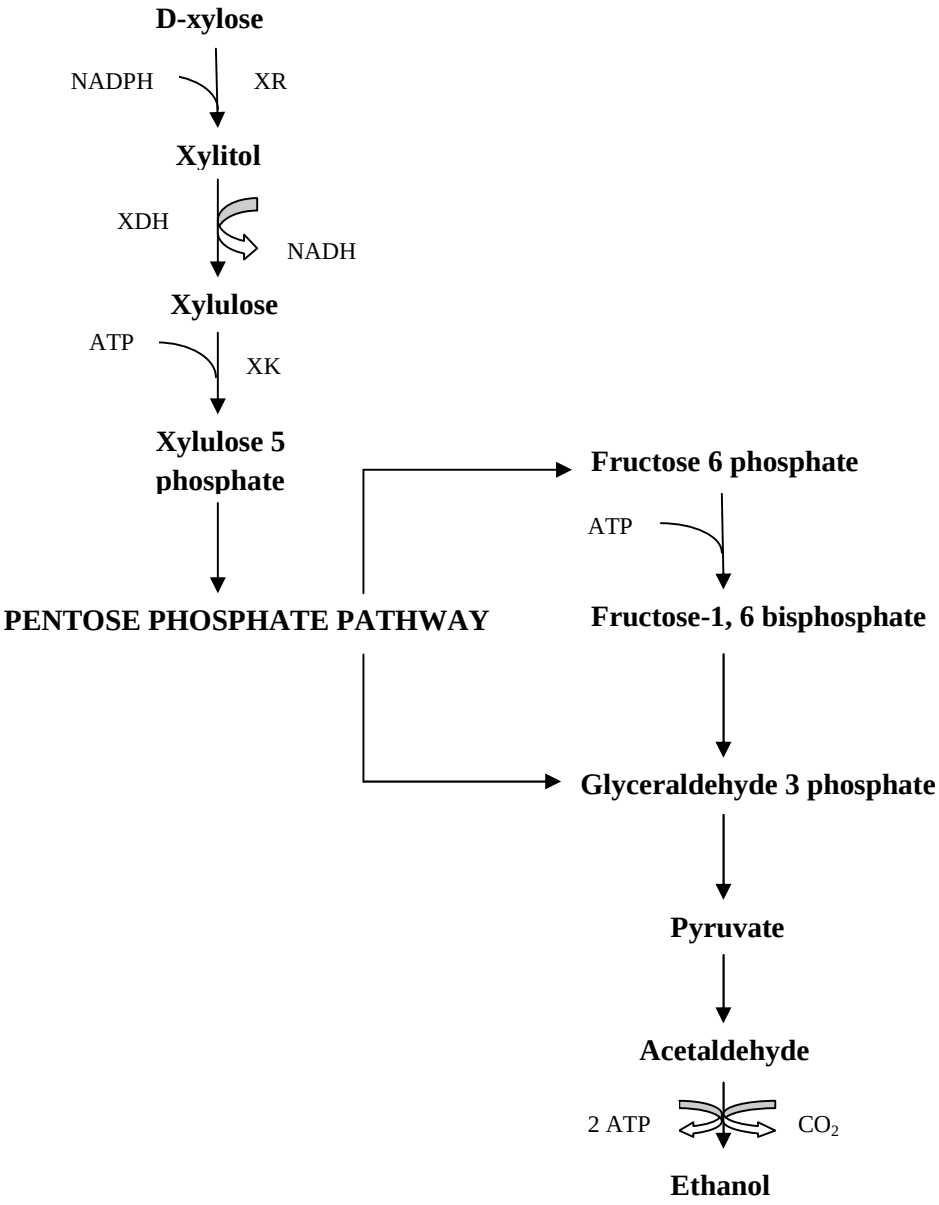


Fig: 6 Xylose Utilization Pathway in Metabolically Engineered *S. cerevisiae* strains. This pathway links D-Xylose metabolism to non-oxidative phase of pentose phosphate pathway and glycolysis **(modified and redrawn from van Maris et al, 2006)**. * XR =Xylose Reductase, XDH = Xylitol Dehydrogenase and XK = Xylulokinase.

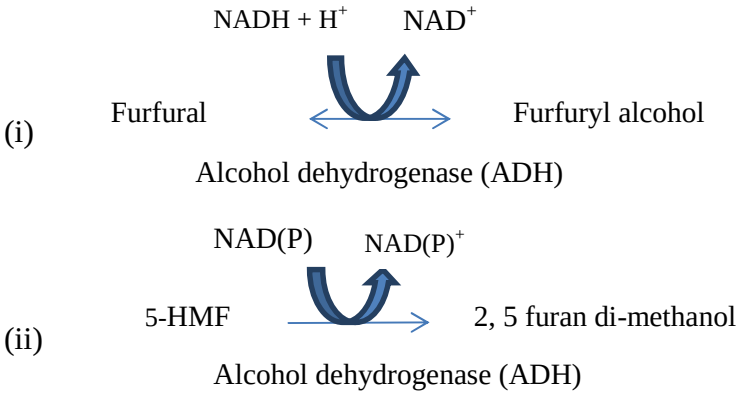
3.5.3.3 Inhibitors and its effects during microbial growth and fermentation process

The high temperature employed during the pre-treatment process generates primary inhibitors such as hydroxymethyl furfural (5-HMF), furfural, hibbert's ketones, vanillins, diphenols and phenyl propane derivatives. At still higher temperatures, primary inhibitors degrade to produce secondary inhibitors: HMF and furfural degrades to produce formic acid (Dunlop, 1948; Ulbricht et al, 1984) and HMF degrades to produce levulinic acid (Ulbricht et al, 1984).

The inhibitors greatly affect the fermentation process by microbial inhibition and they include (i) furan derivatives (2-furaldehyde (furfural) and hydroxymethyl-2-furaldehyde (5-HMF)), (ii) weak acids (mainly acetic acid, formic acid and levulinic acid) and (iii) phenolic compounds (hibbert's ketones, vanillins, diphenols and phenyl propane derivatives) (Palmqvist and Hahn-Hägerdal, 2000)

3.5.3.3.1 Furaldehydes

Furaldehydes are the most potent inhibitors generated during the biomass pretreatment especially during the dilute acid hydrolysis process. In this project, the specific effects of furaldehyde compounds were studied including furfural and 5-HMF. These are chemically related compounds containing a furan ring and an aldehyde group. Furfural is formed by thermal degradation of xyloses (Dunlop, 1948) and 5-HMF is formed by acid catalysed degradation of hexoses (Ulbricht et al, 1984) at higher temperatures. The furan levels might vary depending on the raw material source and the method of pretreatment. Furfural and 5-HMF are metabolized under both aerobic and anaerobic conditions by *S.cerevisiae* (Taherzadeh et al, 1999; Liu et al, 2004). Under anaerobic conditions, *S.cerevisiae* has the ability to reduce 5-HMF and furfural to less toxic alcohols (Villa, 1992; Liu et al, 2004). Furfuryl alcohol/ 2-furanmethanol and 5-hydroxymethyl furfuryl alcohol/ furan-2, 5-dimethanol are formed from furfural (Diaz de Villegas et al, 1992) and 5-HMF, respectively (Liu et al, 2004; Taherzadeh et al, 2000b) by reduction reactions coupled by NADH (Palmqvist et al, 1999) and NADPH-dependent (Wahlbom et al, 2002) alcohol dehydrogenase (ADH). Equation: 3 represent the conversion of furaldehyde compounds to its respective alcohols, namely furfuryl alcohol and 2, 5 furan di-methanol. In aerobic conditions furfural is also oxidized to furoic acid (Sarvari Horvath et al, 2003).



Equation: 3 Conversion of furaldehyde compounds, [furfural (i) and 5-HMF (ii)] to respective alcohols under anaerobic conditions.

Furfural reduction by *S. cerevisiae* under anaerobic condition regenerates NAD^+ resulting in decreased NADH concentration inside the cell (Palmqvist et al, 1999a) which results in decreased glycerol production (Palmqvist et al, 1998). Normally, excess NADH produced during biosynthesis is regenerated by glycerol formation in order to maintain the intracellular redox

balance (Albers et al, 1996; Oura, 1977). Furfural inhibits glycolytic enzymes mainly alcohol dehydrogenase (ADH) (Banerjee et al, 1981). Inhibition of ADH and decreased NADH concentration in the presence of furfural has contributed to increased acetaldehyde accumulation (Palmqvist et al, 1999a) and finally decreased the production of ethanol and acetate. During batch cultivations of *S.cerevisiae*, furfural acts as a strong inhibitor and high concentration results in decreased cell multiplication and longer lag phase due to reduced available cellular energy, decreased total viable cell number and lower volumetric ethanol productivity (Palmqvist et al, 1999; Boyer et al, 1992; Modig et al, 2002). HMF is converted at a very low rate compared to furfural (Taherzadeh et al, 2000b) and slow conversion rate might also cause longer lag phase (Larsson et al, 1998). 5-HMF concentrations in the range of 1-5 g/l affect cell composition, specific growth rate and fermentation rate (Banerjee et al, 1981).

Wahlbom et al, 2002 performed experiments using addition of electron acceptor, acetoin during anaerobic xylose fermentation by recombinant *S. cerevisiae* TMB 3001 to quantitatively measure the response of acetoin by calculating the intracellular fluxes. Other electron acceptors, furfural and hydroxymethylfurfural (5-HMF) was also considered and added during xylose fermentation to study whether these compounds regenerated NAD^+ and lowered xylitol production. After acetoin (6 g/L) addition during fermentation, no xylitol excretion was observed and ethanol yield increased from 0.62mol ethanol/mol xylose to 1.35 mol ethanol/mol xylose compared to the control. After addition of furfural (3 g/L), xylitol and ethanol production were decreased. The addition of 5-HMF (1.5 g/L) resulted in increase of glycerol and acetate production; no decrease in xylitol production; no effect on ethanol and succinate production and exerted a very slow consumption rate compared to furfural.

Taherzadeh et al, 1999 performed anaerobic pulse addition experiments to study the effects of furfural on anaerobic batch cultures of *S.cerevisiae* in glucose. The results indicate that after addition of 4 g/L of furfural to exponentially growing batch cultures, the CO_2 evolution rate (CER) was found to be decreased and the specific ethanol production rate decreased from 1.6(±0.1) to 0.5(±0.2) $g.g^{-1}.h^{-1}$. The specific growth rate μ, decreased from 0.4 to 0.03 (±0.02) h^{-1}. Increased pyruvate formation rate was obtained when furfural was added to the cells and later the rate decreased when the furfural was consumed.

Pulse additions of 5-HMF was performed by Taherzadeh et al, 2000 under anaerobic conditions to study the physiological effects on *S.cerevisiae*. (2-4 g/l) HMF and (2g/l) HMF together with (2g/l) furfural respectively, were added to exponentially growing batch cultures. HMF was mainly converted to 5-hydroxymethylfurfuryl alcohol/ furan 2, 5 di-methanol and 32% decrease in CO_2 evolution rate was observed after pulse addition of 4 g/l HMF. The specific growth rate and specific ethanol production rate decreased from 0.45 h^{-1} to 0.13(±0.00) h^{-1} and 1.6 $g.g.h^{-1}$to 0.95 (±0.05), respectively, after4 g/l HMF addition. In *S.cerevisiae*, HMF is slowly converted to 5-hydroxymethylfurfuryl alcohol due to low membrane permeability and less efficient NADH dependent reduction by alcohol dehydrogenase (Wahlbom et al, 2002). Higher concentrations of 5-HMF decreased the protein content and biomass yield in *S.cerevisiae* (Taherzadeh, 1999). In

simultaneous pulse addition of furfural (2 g/l) and HMF (2g/l), furfural was mainly converted to its respective alcohols before 5-HMF. The specific ethanol production rate was 0.45 (±0.11) $g.g^{-1}.h^{-1}$ and CER decreased by 62% (Taherzadeh et al, 2000).

In general, under anaerobic conditions, the presence of furfural and 5-HMF inhibits microbial metabolism; decrease glucose consumption; decrease xylitol production; increase acetaldehyde accumulation and decrease ethanol production. In yeast, the glucose consumption rate is very low in the presence of high concentrations of furfural and 5-HMF and the decrease in concentration would accelerate glucose consumption towards normal uptake rates (Liu et al, 2004).

3.5.3.3.2 Weak acids

Weak acids have low pH and high pKa values. The undissociated forms of weak acids are liposoluble and diffuse across the plasma membrane from the fermentation medium (Verduyn, 1991). It might dissociate intracellularly and decrease the intracellular pH, which lowers the fermentation rate, inhibits cell proliferation and decreases viability (Verduyn et al, 1990). To maintain a constant intracellular pH, the undissociated forms will continue to enter until equilibrium is attained. The protons present inside the cell are pumped out by the action of plasma membrane ATPase, at the cost of ATP hydrolysis. At high acid concentrations, the proton pumping capacity of the cell is exhausted and increased ATP consumption occurs (Larsson et al, 1999) which in turn provides only little energy for biomass. The anionic forms of acid are hydrophilic and are captured inside the cell and its high concentration results in acidification. In *S.cerevisiae*, low concentration of weak acids exert a stimulating effect on ethanol production by increasing the yield at pH 5.5 and later decreased at higher concentrations (Pampulha and Loureiro-Dias, 1989). Larsson et al, 1998 reported that the same undissociated acid concentration might cause difference in toxicity levels between different carboxylic acids and these variations are due to toxicity difference of anionic form of acids when they enter the cell. The two mechanisms: uncoupling and anion accumulation theory explains the inhibitory effects of weak acids (Russel, 1992).

3.5.3.3.3 Phenolic compounds

The (aromatic) phenolic compounds are formed by lignin degradation and some phenolic compounds are hydroxy-methoxy-benzaldehydes, diphenols/quinines and phenyl propane derivatives. They are usually present in low concentration in the hydrolysates and increases with the harshness of pretreatment process. The most inhibitory phenolic compounds are low molecular weight compounds e.g.: vanillins, 4-hydroxybenzoic acid (Clark and Mackie, 1984). Vanillin is present in large fractions in pine (Clark and Mackie, 1984) and willow (Jönsson et al, 1998) hydrolysates and is less toxic compared to 4- hydroxybenzoic acid (Ando et al, 1986). Phenolic compounds act on biological membranes to affect their potential to serve as selective barriers and enzyme matrices (Heipieper et al, 1994).

3.6 Detoxification

Detoxification is a method employed for specific removal of inhibitors from lignocellulosic hydrolysates prior to fermentation (Olsson, Hahn-Hägerdal, 1996). Different physical, chemical and biological methods are available. Physical detoxification methods include ethyl acetate extraction, roto-evaporation; chemical methods include overliming, sulphite treatment and biological pre-treatments include treatment with the enzymes peroxidase and laccase obtained from *Trametes versicolor* (Jönsson et al, 1998) and treatment with soft-rot fungus *Trichoderma reesei* (Palmqvist et al, 1997). There is no suitable detoxification method available, as there are different lignocellulosic hydrolysates and different microorganisms been used. These detoxification methods reduce inhibitors to a certain extent and complete removal of inhibitors requires some additional process step to be implemented, which might possibly increase the energy requirement of the process. Inhibitor tolerant microorganisms can provide a solution as compared to the detoxification methods described above, in order to make the process simpler and cost effective.

4 MATERIALS AND METHODS

4.1 Yeast strains

Two different recombinant *S. cerevisiae* strains, **GS1.11-26** and **VTT C-10883** were used in this project. In Separate Hydrolysis and Fermentation process (SHF), the cultivations for anaerobic fermentation processes were performed in batch bioreactors using both these strains. **VTT C-10883** was used to perform pulse experiments in anaerobic shake flasks and batch bioreactors**.**

The background of the strain, **GS1.11-26** was from the industrial strain, Ethanol Red, which carries xylose isomerase (*XI*) gene for xylose utilization and later UV mutagenized and evolutionary engineered to improve the utilization of xylose. The strain was maintained on YEPD/YPD (Yeast Extract Peptone Dextrose) plates, incubated at 4°C and stock cultures were stored at -80°C.

VTT C-10883 strain has been constructed by over expressing the endogenous xylulokinase encoding gene (*XKS1*) and integrating xylose reductase (XR)/*XYL1* and xylitol dehydrogenase (XDH)/*XYL2* encoding genes of *S. stipitis* in CEN.PK 113-1A background. The strain was maintained on YEPD/YPD (Yeast Extract Peptone Dextrose) plates incubated at 4°C and stock cultures were stored at -80°C.

4.2 Media for pulse experiments

4.2.1 Defined mineral medium

Pulse experiments in anaerobic shake flasks were carried out using VTT C-10883 grown in defined mineral medium containing per litre: glucose (20 g), xylose (20 g) and salts: 7.5 g $(NH_4)_2SO_4$, 3.5 g KH_2PO_4 and 0.75g $MgSO_4.7H_2O$ (Verduyn et al,1990). In addition, ergosterol (10mg) and Tween (420 mg) were added to the defined medium to maintain anaerobic growth (Appendix: B). Pulse experiments in anaerobic batch bioreactors were also carried out using VTT C-10883 grown in defined mineral medium containing per litre: glucose (40 g); xylose (50 g), salts: 7.5 g $(NH_4)_2SO_4$, 3.5 g KH_2PO_4 and 0.75 g $MgSO_4.7H_2O$, ergosterol(10 mg) and tween 20 (420 mg) (Appendix: C)

4.2.2 Inhibitors

In pulse experiments, a mixture of HMF and furfural was used along with the defined mineral medium to analyse the stress response of the VTT C-10883 strain. The preliminary experiment carried out in anaerobic shake flasks uses three different inhibitor concentrations.The stress response of VTT C-10883 strain was further analysed on up-scaled conditions by performing pulse experiments using high inhibitor concentration in batch bioreactors.

Table: 2 Different inhibitor concentrations (g/L) used in pulse experiments.

Inhibitor concentration	HMF	Furfural
Low	1.3	0.4
Medium	2.6	0.8
High	3.9	1.2

4.3 Separate Hydrolysis & Fermentation (SHF) process - Experimental set up

In SHF, the hydrolysis and fermentation process were carried out separately as two different steps. In the hydrolysis step, each pretreated lignocellulosic material (spruce or *Arundo*) was hydrolysed separately with the help of enzymes and the resulting liquid hydrolysate of each material was used in the fermentation process separately.

4.3.1 Substrate and Enzyme mixtures for Enzymatic Hydrolysis

4.3.1.1 Substrate: *spruce and Arundo donax*

The pretreated lignocellulosic materials such as spruce and *Arundo* were obtained from SEKAB, Örnsköldsvik-Sweden and Chemtex-Italy, respectively. The composition of the materials estimated by the company is represented in Table: 3. It was stored at -20°C in containers and later for immediate use in the experiments they were stored at 4°C.

Table: 3 Material compositions (g/L) of the pretreated raw materials used in SHF

	Arundo donax[1]	Spruce[2]
SOLID FRACTION[3]		
Glucose	55.6	67.5
Xylose	8.1	0.3
Mannose	-	0.4
LIQUID FRACTION		
Glucose	8.5[4]	26.0
Xylose	17.1[4]	8.0
Mannose	-	18.0
Galactose	-	3.4
Acetic acid	2.6	4.3
Formic acid	n.a	0.2
HMF	0.4	3.3
Furfural	0.2	1.7

[1]Analyzed by Chemtex
[2]Analyzed by SEKAB
[3]Corresponding to monomeric sugars after hydrolysis
[4]Present as oligomers in the liquid
n.a = not analyzed.

4.3.1.2 Enzyme mixtures

In enzymatic hydrolysis, each pretreated lignocellulosic material was hydrolysed with different enzyme mixtures (Table: 4). In the hydrolysis of spruce, Thermomix enzymes obtained from Roal, Finland was used. Thermomix include Cellobiohydrolases I, Cellobiohydrolases II, Endoglucanases II and β-Glucosidase. In the hydrolysis of *Arundo*, Celluclast and Novozyme 188 obtained from Novozymes, Denmark; Xyl11 obtained from Dyadic, Netherlands was used.

Table: 4 Different enzyme mixtures used for enzymatic hydrolysis.

S.No	METHOD	ENZYME MIXTURES
1	Enzymatic hydrolysis of spruce	Cellobiohydrolases (CBH) I - 3.6 mg/g DW Cellobiohydrolases (CBH) II - 4.9 mg/g DW Endoglucanases (EG) II - 1.5 mg/g DW Beta Glucosidase (β-G) - 1.5 mg/g DW
2	Enzymatic hydrolysis of *Arundo*	Celluclast - 9 mg/g WIS Xyl11 - 1 mg/g WIS Novozyme 188 - 500 nkat/g WIS

4.3.1.3 Enzymatic Hydrolysis

In enzymatic hydrolysis, each pretreated material was hydrolysed with different enzyme mixture (Table: 4) and performed in triplicates. This process was carried out in Infors reactors with a working weight of 3 kg in each reactor. The final WIS (Water Insoluble Solids) concentration of the pretreated material was 13% in each reactor and pH of the material was set to 5 using 12 M NaOH. The process temperature and stirrer speed were set to 45° C and 500 rpm, respectively. The pH was maintained at 5 using 12 M NaOH, manually and corresponding enzymes were added to each pretreated material when the temperature reached 45° C. Samples were taken at regular intervals: before enzyme addition: 0 hours, after enzyme addition: 24, 48 and 72 hours. 2 samples of each 2 ml volume were taken from each reactor using pipette and were heated at 100°C in a boiling water bath for 10 minutes to inactivate the enzymes. The samples were then centrifuged at 14000 rpm for 2 minutes. After filtering through a 0.2 µm nylon membrane, the supernatant was collected in eppendorf tubes and stored in the freezer until HPLC analysis.

4.3.2 Fermentation

The fermentation step in Separate Hydrolysis and Fermentation process (SHF) was carried out in both anaerobic shake flasks and bioreactors.

4.3.2.1 Anaerobic fermentation in shake flasks

In anaerobic shake flasks, the fermentation process were carried out using two different strains: VTT C-10883 and GS1.11-26 cultivations on two different liquid hydrolysates: spruce and *Arundo* obtained from enzymatic hydrolysis. 12 anaerobic shake flasks of 200 ml volume each were prepared. Among 12, 6 shake flasks of each 100 ml working volume were used to test the growth and fermentation performance of each strain on two different liquid hydrolysates. Each strain and material were analysed in triplicates.

Each hydrolysate (spruce or *Arundo*) was supplemented with 1 g/L yeast extract, 0.5 g/L $(NH_4)_2HPO_4$ and 0.025 g/L $MgSO_4.7H_2O$. 4 g/L (dry weight) inoculum was added to each shake flask and later these flasks were incubated at 30°C in a rotary shaker at 120 rpm. Samples for OD measurement and HPLC analysis were taken at regular intervals: 0, 2, 4, 6, 8, 10, 24, 48, 72 and 96 hours. During sampling, 2 ml was withdrawn each time from each shake flask: 0.5 ml was used to measure OD at 650 nm, 0.5 ml was centrifuged and supernatant was used as a blank for OD measurement and 1 ml was filtered using 0.2 µm nylon membrane and stored in the freezer until HPLC analysis.

4.3.2.2 Anaerobic batch fermentation in bioreactors

In anaerobic batch fermentation process, the strain GS1.11-26 was used to ferment each liquid hydrolysate: spruce and *Arundo*. This fermentation process requires larger amount of cells that were cultivated by different cultivation methods: shake flask cultivation, aerobic batch cultivation, and fed-batch cultivation on each hydrolysate. The cells obtained from the fed-batch cultivation on each hydrolysate were harvested and used for its corresponding anaerobic batch fermentation process.

4.3.2.2.1 Propagation of cells

4.3.2.2.1.1 Inoculum cultivation (shake flask)

GS1.11-26 strain was inoculated in 2 shake flasks containing 250 ml defined mineral medium with glucose (Appendix: C). These flasks were incubated at 30°C at 105 rpm for 20-24 hours.

4.3.2.2.1.2 Aerobic batch cultivation

Aerobic batch cultivation was carried out in Infors reactors with a working volume of 0.7 L. The medium (Appendix: C) was taken in the reactor and pH was maintained at 5 by automatic addition of 2 M NaOH. The other parameters that were maintained include: temperature- 30°C, stirrer speed-800 rpm, air flow rate- 1.2 L/minute. The cells were cultivated for 20-22 hours.

4.3.2.2.1.3 Fed-batch cultivation

Fed-batch cultivation was initiated after 20-22 hours of aerobic batch cultivation. In this cultivation method, 1 L of each pretreated liquid was pumped automatically to the reactor. The feed rate was initially 40 mL/h and was increased linearly to 100 mL/h. Fed-batch cultivation method was carried out in order for the cells to get adapted to the process conditions and consume the pretreated liquid. The parameters that were controlled during this cultivation include: temperature- 30°C, stirrer speed- 1000 rpm, air flow rate- 1.5 L/minute. The cells were cultivated for 16 hours.

Note: Each homogenised pretreated lignocellulosic material was centrifuged at 10,000 rpm for 10 minutes and the supernatant was collected in a flask. The filtered liquid supernatant (pretreated liquid) was adjusted to pH 5 using 12 M NaOH. This liquid was then sterile filtered and used in the fed-batch cultivation.

4.3.2.2.2 Cell harvest and anaerobic batch fermentation

4.3.2.2.2.1 Cell harvest

Samples collected from fed-batch cultivation were centrifuged at 1800 g in 500 mL centrifuge bottles and the pellets were resuspended in 0.9% NaCl solution. Samples for dry weight measurement were taken from the cell suspension and total cell mass concentration was adjusted to 80 g/L using 0.9 % NaCl.

Note: The time between the end of fed batch cultivation and addition of harvested cells to the fermentor must be no longer than 3 hours.

4.3.2.2.2.2 Anaerobic batch fermentation

Anaerobic batch fermentation process was carried out using each liquid hydrolysate obtained from its corresponding enzymatic hydrolysis. Each liquid hydrolysate was analysed in duplicates and working volume in each reactor was 1400 ml. The hydrolysates were supplemented with 1 g/L yeast extract, 0.5 g/L $(NH_4)_2HPO_4$, 0.025 g/L $MgSO_4.7H_2O$ and 500 µL antifoam. The fermentation was initiated by adding cells at a final concentration of 4 g/L (dry weight) to each fermenter. These batch cultivations were performed at 32°C with a stirrer speed of 500 rpm. The pH was maintained at 5 using automatic addition of 2 M NaOH.

The cultivations were run for 96 hours and samples were withdrawn at regular intervals: 0, 2, 4, 6, 8, 10, 24, 48 and 96 hours for dry weight measurement and HPLC analysis.

4.4 Pulse experiment set up

4.4.1 Different cultivation methods for pulse experiments

4.4.1.1 Anaerobic cultivations in shake flasks

Anaerobic shake flasks were prepared by inserting a glass water lock into a rubber stopper fitted to a 200 ml shake flask containing 3 baffles. Half of the glass water lock was filled with 99.9 % glycerol and the opened end of it was covered with an aluminium foil. A short tube was also inserted into the rubber stopper; one end of the tube goes to the bottom of the shake flask and to the other end, a syringe was attached to take samples. Samples were taken using a syringe attached to a short tube that was inserted into the shake flask through a rubber stopper. In these shake flasks, VTT C-10883 strain was grown using defined mineral medium (Appendix: D) containing both glucose and xylose (and these cultures were pulsed with three different inhibitor concentrations.

12 anaerobic shake flask cultures were prepared, among which 6 shake flask cultures were used for pulse experiments during exponential growth phase (glucose) and the remaining 6 shake flask cultures were used for pulse experiments during xylose phase. The shake flask cultures prepared for xylose pulse experiments were incubated at 30˚ C in a rotary shaker at 150 rpm till it reached xylose phase (approximately, 27-29 hours). For pulse experiments during exponential growth phase (glucose), the three different inhibitor concentrations were pulsed when OD was around 1

and for pulse experiments during xylose phase, the three different inhibitor concentrations were pulsed during xylose consumption phase. All anaerobic shake flask cultures were incubated at 30°C in a rotary shaker at 150 rpm and samples were taken at regular intervals. During sampling, 2ml sample volume was withdrawn each time using syringe, among which 1 ml was used to measure OD at 600 nm and remaining 1 ml sample was filtered using 0.2 µm nylon membrane filter and stored in the freezer until HPLC analysis.

4.4.1.2 Anaerobic cultivations in batch bioreactors

The pulse experiments were also carried out in batch bioreactors. VTT C-10883 strain was grown under anaerobic conditions using defined mineral media (Appendix: D) in DASGIP fermenters with a working volume of 1.5 liters. 0.1 VVM (Volume per Volume per Minute) of nitrogen gas was sparged into the fermenter and pH was maintained at 5 using 2M NaOH throughout the fermentation process. The stirring rate of the impeller was kept at 200 rpm and temperature was maintained at 30°C.

Four different experiments were performed in the reactors: glucose control (addition of no inhibitors), addition of high concentration inhibitor pulse during exponential growth phase (glucose), xylose control (addition of no inhibitors) and addition of high concentration inhibitor pulse during xylose phase. In glucose pulse experiment, high concentration inhibitor was pulsed in mid exponential phase (OD was around 2) and in xylose pulse experiment, it was pulsed after four hours of complete glucose uptake. Each reactor experiment was performed in triplicates. Samples were taken at regular time intervals to measure OD at 600nm, analyse extracellular metabolites in HPLC and dry weight measurements. In all the experiments, samples were taken for both intracellular (adenonucleotides) and extracellular metabolites (glucose, xylose, xylitol, ethanol, glycerol, HMF, furfural) at different time intervals.

The extracellular metabolite samples were taken at different time intervals i.e., in glucose control experiment, samples were taken at 0 hour (lag phase), 12, 14, 16, 18, 20 hours (in exponential growth phase-glucose phase) and at different time periods in stationary phase until 92 hours. In xylose control experiment, samples were taken at 0 hour, during exponential growth phase and at 22, 24, 26, 30 hours (during xylose phase) and at different time periods until 89 hours. In glucose pulse experiment, samples were taken at 0 hour, each hour after pulsing during exponential growth phase and at varied time periods in stationary phase until 88 hours. In xylose pulse experiment, samples were taken at 0 hour, in exponential growth phase and each hour after pulsing during xylose phase (continued till 6 hours after pulsing) and at varied intervals after 6 hours of pulsing until 88 hours.

In glucose control and xylose control, intracellular metabolite samples were taken when the OD was around 2 and after four hours of complete glucose uptake, respectively. In glucose pulse and xylose pulse experiment, samples were taken after one hour of pulse for intracellular metabolite analysis.

4.5 Analytical measurements

4.5.1 Optical Density (OD)

Pulse experiment samples and samples from SHF process in anaerobic shake flasks were measured for OD at 600 nm and 650 nm, respectively using Genesys 20 spectrophotometer.

4.5.2 Dry weight determination

Samples from pulse experiments and SHF process in batch bioreactors were used to measure dry weights. It was performed using PESU (polyethersulphone) membrane filters of 0.2 µm and 4 cm diameter, (Sartorius Biolab). These membrane filters were numbered, pre-dried in a microwave oven at 126 W for 15 minutes and kept in a desiccator for few hours before they were weighed. These measurements were performed in duplicates for all samples and for each measurement, the membrane filter was kept in a vacuum sucker (water driven).The filter was pre-wet 2-3 times with milli-Q water.5 ml of sample culture was added slowly to the centre of the filter and again washed 2-3 times with milli-Q water. The filters were vacuum dried for 30sec to 1 min after which they were dried in the microwave oven at 126 W for 15 min. The dried filters were put inside a desiccator and left overnight to dry completely. Lastly, the filter was again weighed.

4.5.3 HPLC-Extracellular metabolite analysis

High performance liquid chromatography (HPLC) was used to measure sugars and extracellular metabolites: glucose, xylose, cellobiose, arabinose, xylitol, mannose, glycerol, acetate, succinate, pyruvate, ethanol, HMF and furfural. Glucose, xylose, cellobiose, arabinose, mannose, xylitol, ethanol, glycerol and succinate were detected using RI detector and other compounds such as HMF, furfural, acetate and pyruvate were measured using UV detector at 210 nm.

The pulse experiments (both shake flasks and bioreactors)and SHF samples from both enzymatic hydrolysis and fermentation (both shake flasks and bioreactors) were analysed in HPLC using Aminex HPX-87H column maintained at 60°C and Aminex HPX-87P column maintained at 85°C, respectively. 5mM $H_2SO_4$was used as eluent at a flow rate of 0.6 ml/ min. The following compounds, glucose, xylose, glycerol, xylitol, ethanol, HMF and furfural were analysed in these samples. Apart from these compounds, sugars such as cellobiose, mannose, arabinose and galactose were also analysed in SHF samples (both enzymatic hydrolysis and fermentation).

4.5.4 Intracellular metabolite analysis

Intracellular metabolite samples for adenonucleotides were also analysed in HPLC to determine AXP (ATP/ADP/AMP) concentrations

4.5.4.1 Sampling and extraction procedure for ATP, ADP and AMP

In pulse experiments, during sampling for AXP (ATP/ADP/AMP), eight pre-weighed falcon tubes were used for each reactor, so in total 24 tubes were used for each experiment. From each reactor, a sample volume of about 60 mL was withdrawn, among which 5 mL (in total 8 tubes for each reactor * 5 mL) was used for this measurement and remaining was used for dry weight,

OD and HPLC measurements. Later 24 tubes were centrifuged at 5000 g for 5 minutes at -20 ˚ C after which the supernatant was removed and the pellets were frozen in liquid N_2. The samples were later stored at -80˚C until analysis.

4.5.4.1.1 Extraction of ATP/ADP/AMP with TCA

Each cell pellet was dissolved in 0.5 mL of 0.51 M trichloroacetic acid (TCA) containing 17 mM EDTA. These cells were allowed to vortex for 5 seconds and placed in an ice bath for 15 minutes. Vortex was again carried out in the middle and at the end of incubation. Later these extracts were transferred to eppendorf tubes and centrifuged at a maximum speed for 3 minutes at 4 ºC. The contents were transferred to a pre-weighed eppendorf tubes and the volume of each extract were noted down. Each extract was then neutralised by adding 0.29 mL 2M Tris Base (pH 10.8) per mL extract and later these samples were analysed using HPLC.

HPLC (Ultimate 3000, Dionex Corp., Sunnyvale, US) equipped with a quaternary analytical pump HPG-3400A (Dionex Corp., Sunnyvale, US) and fitted with Luna® 5u C18 (2) 100Å LC column (150 x 4.6 mm) (Phenomenex Inc., Torrance, US) kept at 20°C was used to determine the concentrations of adenosine nucleotides.The mobile phase consisted of acetonitrile and tetrabutylammonium buffer (0.005 M tertrabutylammonioum hydrogensulfate, 0.01 M Na_2HPO_4), pH 7.0. The time intervals for acetonitrile gradient were as follows: t0 min 6%, t3 min 6%, t16 min 25%, t22 min 25%, t27 min 6% and then the system was equilibrated for 8 minutes to the initial conditions. The flow rate was 1 ml/min. The detection was performed using photodiode array detector PDA-3000 (Dionex Corp., Sunnyvale, US) at 260 nm. Peak identities were confirmed by co-elution with standards and quantification was carried out by comparison using standard solutions of known concentrations.

4.5.5 WIS (Water Insoluble Solids) measurement

The homogenised pretreated lignocellulosic material of spruce and *Arundo* were used to measure WIS concentration. Each pretreated material was added to a pre-weighed falcon tube and the resulting mass were noted down. Each material was analysed in triplicates and these falcon tubes were centrifuged at 10,000 rpm for 10 minutes. After centrifugation, the supernatant was discarded and 5 ml of distilled water was added to each falcon tube and mixed well for 3-4 minutes. These tubes were centrifuged and washed 4-5 times until the glucose present in the material was completely gone. The lids of these tubes were removed and the tubes were kept in oven overnight at 105° C. Later the weight of the tubes with dried material (referred as ODW-Oven Dry Weight) was noted down.

4.6 Analytical calculations

4.6.1 Specific growth rate

The specific growth curve was obtained by plotting ln (OD) values against time.

In pulse experiments, different data points [ln (OD)] were considered for each experiment to calculate specific growth rates. In glucose control experiment (without inhibitor addition), the

data points in the exponential phase were considered and plotted against time. In pulse during glucose phase (exponential phase) and pulse during xylose phase, the data points after pulsing were considered and plotted against time. For xylose control experiment (without inhibitor addition), the data points in the xylose consumption phase were considered and plotted against time. Thus, specific growth rate was calculated by taking slope of corresponding data points in each experiment.

4.6.2 Average yields

In pulse experiments, average yields of product (ethanol, xylitol, glycerol, acetate and biomass) were calculated using the given formula.

$$\textit{Avg. ethanol yield} = \frac{Avg.\ amount\ of\ ethanol\ produced\ at\ the\ end\ of\ fermentation}{Avg.amount\ of\ consumed\ sugars}$$

$$\textit{Avg. xylitol yield} = \frac{Avg.amount\ of\ xylitol\ produced\ at\ the\ end\ of\ fermentation}{Avg.amount\ of\ consumed\ xylose}$$

$$\textit{Avg. glycerol yield} = \frac{Avg.amount\ of\ glycerol\ produced\ at\ the\ end\ of\ fermentation}{Avg.amount\ of\ consumed\ sugars}$$

$$\textit{Avg. acetate yield} = \frac{Avg.amount\ of\ acetate\ produced\ at\ the\ end\ of\ fermentation}{Avg.amount\ of\ consumed\ sugars}$$

$$\textit{Avg. biomass yield} = \frac{Avg.\ amount\ of\ biomass\ produced\ at\ the\ end\ of\ fermentation}{Avg.\ amount\ of\ consumed\ sugars}$$

In enzymatic hydrolysis, average glucose/xylose yields were calculated using the given formula

$$\textit{Avg. sugar yield} = \frac{Avg.\ amount\ of\ sugar\ released\ at\ the\ end\ of\ hydrolysis}{Amount\ of\ sugar\ present\ in\ the\ solid\ fraction\ of\ the\ pretreated\ material}$$

*Avg- Average.

4.6.3 Specific inhibitor conversion rates

Specific inhibitor (HMF/ Furfural) conversion rate =

$$\frac{Inhibitor\ concentration\ difference\ (Ci-Cf)}{Avg.cell\ concentration\ (ODi,ODf)*Time\ difference\ for\ inhibitor\ concentration\ (\Delta t)}.$$

4.6.4 Dry weight concentration

The dry weight for each sample was calculated by taking difference of two weights (filter weight after use and filter weight before use) and represented in g/L.

4.6.5 Determination of Energy Charge

The energy charge was calculated using the formula

(i) Energy charge E_c =[ATP] + 0.5 [ADP]/ [ATP] + [ADP] + [AMP]

4.6.6 Determination of WIS concentration

The amount of Fraction Insoluble Solids in the Slurry (FISS) was calculated by dividing the oven dry weight (ODW) of the washed solids with the weight of the slurry (difference between weight of the falcon tube with slurry and weight of the empty falcon tube).

$$\%FISS\ (Fraction\ Insoluble\ Solids\ Slurry) = \frac{ODW_{washedsolids}}{Weight_{bottle,cap,\&slurry} - Weight_{bottle\&cap}} \times 100$$

5 RESULTS

In this section, the results obtained from Separate Hydrolysis and Fermentation process (SHF) and Pulse experiments are reported.

5.1 Separate hydrolysis and fermentation (SHF)

5.1.1 Enzymatic hydrolysis

In order to release the fermentable sugars from the pretreated lignocellulosic material, enzymatic hydrolysis was carried out. In this hydrolysis, each pretreated material: spruce and *Arundo* was hydrolyzed with the help of two different enzyme mixtures. As mentioned earlier, Cellobiohydrolases (CBH) I, Cellobiohydrolases (CBH) II, Endoglucanases (EG) II and Beta Glucosidase (β-G) were the enzymes used in the hydrolysis of spruce and in the hydrolysis of *Arundo*, the enzymes Celluclast, Xyl11 and Novozymes 188 were used. From the material composition of the pretreated material (Table: 3) we observed that *Arundo* had more amounts of xylose sugar present in both solid and liquid fraction compared to spruce. So the enzyme, xylanase was added to the enzyme mixture that we used for *Arundo*. The hydrolysis was carried out in triplicates for 72 hours.

Fig: 7 represents average quantitative data: glucose and xylose obtained from enzymatic hydrolysis of spruce **(a)** and *Arundo* **(b)**. In the hydrolysis of spruce, the average sugar and inhibitor concentrations at the end of 72 hours with 13% WIS were glucose: 77.3 (±1.4) g/L, xylose: 7 (±0.03) g/L, galactose: 3.2 (±0.01) g/L, arabinose: 2.2 (±0.006) g/L, mannose: 14.8 (±0.05) g/L, HMF: 3 (±0.03) g/L and furfural: 2 (±0.04) g/L. The average glucose yield was calculated to be 0.87 g/g by dividing the amount of particular sugar released at the end of hydrolysis to the amount of particular sugar (g/L) present in the solid fraction of the material.

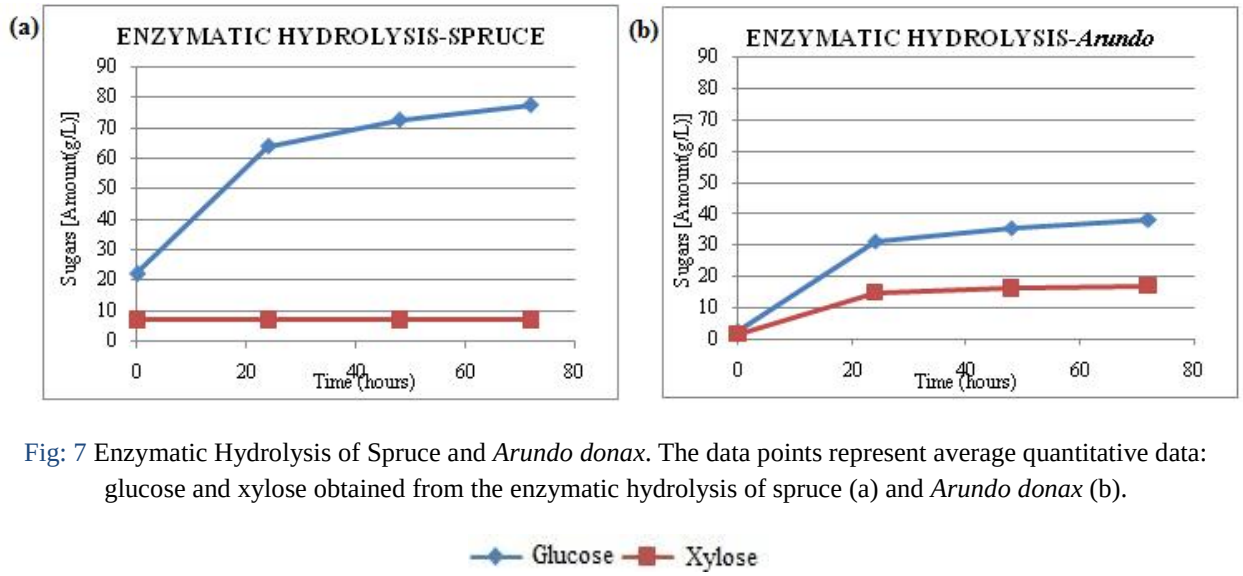


Fig: 7 Enzymatic Hydrolysis of Spruce and *Arundo donax*. The data points represent average quantitative data: glucose and xylose obtained from the enzymatic hydrolysis of spruce (a) and *Arundo donax* (b).

In the hydrolysis of *Arundo*, the average sugar and inhibitor concentrations at the end of 72 hours with 13% WIS were glucose: 38(±1.1) g/L; xylose: 17 (±0.1) g/L; galactose: 1.7 (±0.02) g/L; arabinose: 1 (±0.0) g/L; mannose: 1.7 (±0.02) g/L, HMF: 0.4 (±0.0) g/L and furfural: 0.2(±0.01) g/L. The average glucose (0.53g/g) and xylose (0.63g/g) yields were calculated by dividing the amount of sugar released at the end of hydrolysis to the sum of corresponding sugar (g/L) present in the solid fraction and its oligomers present in the liquid fraction.

5.1.2 Anaerobic fermentation

5.1.2.1 Anaerobic fermentation in shake flasks

Anaerobic cultivations of GS1.11-26 and VTT C-10883 strains were carried out to perform preliminary fermentation experiments in shake flasks using two different liquid hydrolysates obtained from enzymatic hydrolysis. The main objective of carrying out fermentation in anaerobic shake flasks was to estimate and compare the fermentation performance of each strain: GS1.11-26 and VTT C-10883 on two different liquid hydrolysates: spruce and *Arundo*. These two strains have different xylose utilization pathways, i.e., GS1.11-26, an industrial strain carries *XI* gene and VTT C-10883, a laboratory strain carries *XR* and *XDH* genes for xylose utilization. The sugar composition was different in each liquid hydrolysate: more amounts of glucose were present in spruce compared to *Arundo* and on the other hand, more amounts of xylose were present in *Arundo* compared to spruce.

The fermentation process of spruce and *Arundo* were run for 96 hours. Each strain and liquid hydrolysate was analyzed in duplicates. Fig: 8 represent the average quantitative data: glucose, xylose/galactose/mannose, ethanol and cell concentration (OD_{650}) obtained from spruce fermentation using the strain GS1.11-26 **(a)** and VTT C-10883 **(b)**.

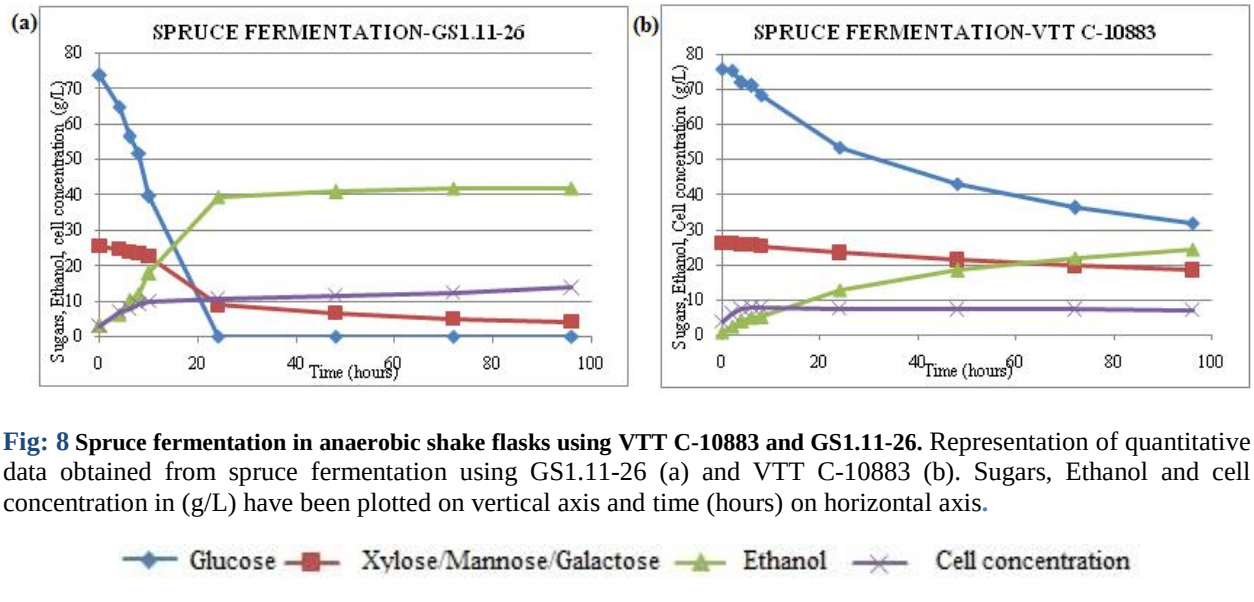


Fig: 8 Spruce fermentation in anaerobic shake flasks using VTT C-10883 and GS1.11-26. Representation of quantitative data obtained from spruce fermentation using GS1.11-26 (a) and VTT C-10883 (b). Sugars, Ethanol and cell concentration in (g/L) have been plotted on vertical axis and time (hours) on horizontal axis.

In spruce fermentation, GS1.11-26 strain consumed all the sugars and glucose was completely exhausted before 24 hours. During these hours, rapid consumption of glucose was observed and

xylose consumption started after glucose depletion at a linear rate, slowly decreasing with time. The amount of xylose/galactose/mannose left over were 4.2 g/L at 96 hours. Due to high amount of consumed sugars, higher cell concentration (OD_{650}) 13.8 and ethanol concentration (42 g/L) were obtained at 96 hours. Lower amounts of glycerol were produced, 4.2 g/L reflecting that more amounts of glucose were used only for ethanol production and the average ethanol yield was calculated to be 0.41 (±0.0) g ethanol produced at the end of fermentation/g consumed sugars.

VTT C-10883 strain in spruce fermentation consumed only half the amount of glucose and xylose/galactose/mannose compared to GS1.11-26. At 96 hours, 31.7 g/L of glucose and 18.4 g/L of xylose/galactose/mannose were still present in the fermentation medium. Due to consumption of lower amounts of sugars, the resulting cell concentration (OD_{650}) and ethanol concentration were low, 7.2 and 24.4 g/L, respectively. Lower amounts of xylitol were produced due to redox imbalance during xylose conversion step (Kötter and Ciriacy, 1993).

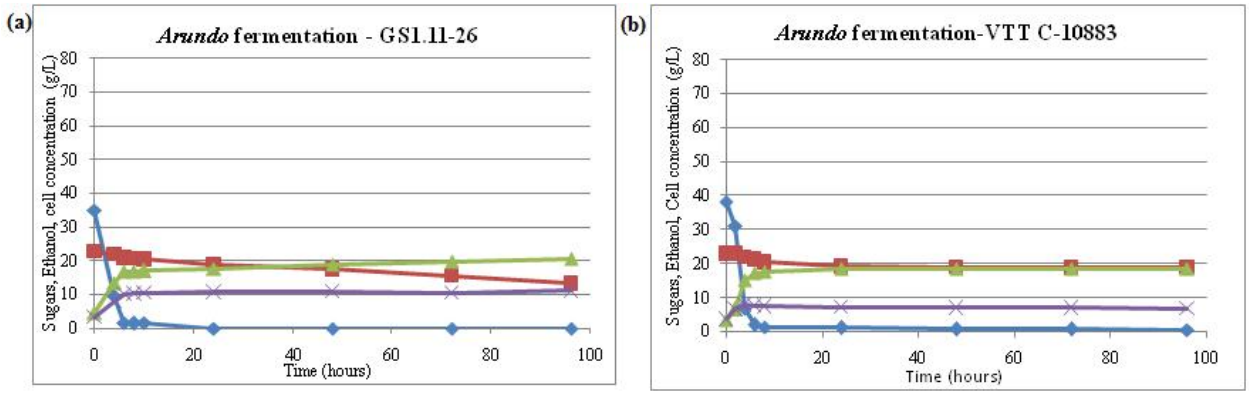


Fig: 9 ***Arundo*** **fermentation in anaerobic shake flasks using VTT C-10883 and GS1.11-26.** The data points represent the average quantitative data: glucose, xylose, ethanol and cell concentration obtained from *Arundo* fermentation using the strain GS1.11-26 (a) and VTT C-10883(b).

In *Arundo* fermentation (Fig: 9), GS1.11-26 strain **(a)** consumed glucose completely during the first 11-12 hours. In comparison to the spruce fermentation, GSI.11-26 strain consumed xylose at a slower rate and the amount of xylose/galactose/mannose left over was 13.2 g/L at 96 hours. The cell concentration (OD_{650}) and ethanol concentration of the strain were 11.2 and 20.6 g/L, respectively at 96 hours. 2.3 g/L glycerol was produced at the end of fermentation and ethanol yield was calculated to be 0.36(±0.03) g ethanol produced/g consumed sugars.

VTT C-10883 strain **(b)** does not consume all the sugars, 0.5 g/L glucose and 18.8 g/L xylose/galactose/mannose were left over at the end of fermentation. Due to consumption of lower sugars, the resulting ethanol concentration was low, 18.7 g/L and ethanol yield was calculated to be 0.4 (±0.0) g ethanol produced/g consumed sugars. VTT C-10883 strain was able to grow only in the glucose consumption phase and at the end of fermentation, a cell

concentration of 6.8 (OD_{650}) was obtained. 1.6 g/L xylitol was produced during the xylose conversion.

At the end of 96 hours in both Spruce and *Arundo* fermentation, viability measurements were performed on YPD plates in triplicates. 10^4 and 10^5 dilutions were made using the fermentation samples and number of colonies was calculated (Table: 5).

Table: 5 Cell colonies (GS1.11-26 and VTT C-10883) present at the end of both Spruce and Arundo fermentation (96 hours)

- GS1.11-26

Spruce	Arundo
10^4- 115 (±0.9)colonies	10^4 – 198 (±1.2) colonies
10^5 – 18 (±0.36)colonies	10^5- 128 (±0.4) colonies

- VTT C-10883

Spruce	Arundo
10^4- 2 (±0.15) colonies	10^4 – 92 (±0.27) colonies
10^5 - 0 colonies	10^5- 54 (±0.19) colonies

*quantitative data represented in the above table were obtained from triplicate measurements

5.1.2.2 Anaerobic fermentation in batch bioreactors

The fermentation experiments performed in anaerobic shake flasks using GS1.11-26 strain showed significant rise in cell concentration (OD_{650}), ethanol concentration and sugar consumption, compared to VTT C-10883. So GS1.11-26 strain was considered for the fermentation experiments in batch bioreactors in order to analyze the strain performance in up-scaled conditions and to measure dry weights. The fermentations were initiated by adding cells at a concentration of 4 g/L to each reactor and the cultivations were run for 96 hours. The inoculum prepared *Arundo* fermentation did not contain enough less number, so the fermentation was initiated by adding cells at a concentration less than 4 g/L. Fig: 10 represents average quantitative data: glucose, xylose, ethanol and dry weight concentration obtained from spruce **(a)** and *Arundo* **(b)** fermentation.

In spruce fermentation, GS1.11-26 strain rapidly consumed both glucose and xylose; xylose was completely depleted at 48 hours, approximately. Galactose (3.1 g/L), arabinose (2.1 g/L) and mannose (14.4 g/L) were also present in liquid hydrolysate. Mannose was completely consumed and very low amounts of galactose (0.6 g/L) and arabinose (1.5 g/L) were left at 96 hours. More amounts of ethanol were produced during the glucose consumption phase and due to the consumption of all sugars, higher ethanol concentration (41.4 g/L) was observed at the end of fermentation. Based on the consumed sugars, the average ethanol yield was calculated to be 0.42

(±0.01) g ethanol produced/ g consumed sugars. Glycerol was produced during glucose consumption phase as a consequence of biomass production (Zaldivar et al., 2005) and 5.1 g/L glycerol was obtained at 96 hours, indicating that most of the glucose was used for the production of ethanol. 5.1 g/L dry weight concentration was obtained at 96 hours.

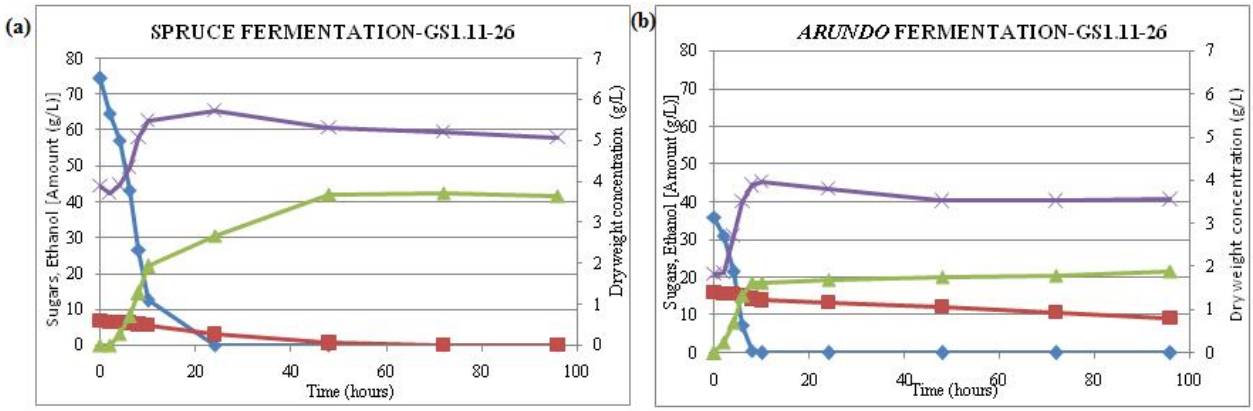


Fig: 10 Spruce and *Arundo* fermentation using GS1.11-26. The data points represent the average quantitative data: glucose, xylose, ethanol and dry weight concentration obtained from spruce (a) and *Arundo* (b) fermentation using the strain, GS1.11-26.

In *Arundo* fermentation, the cells consumed all glucose and 9.3 g/L xylose was still present in the fermentation medium at 96 hours. Other sugars present in the liquid hydrolysate include galactose (1.6 g/L), arabinose (0.9 g/L) and mannose (1.6 g/L). At the end of fermentation, 21.8 g/L ethanol was produced and average ethanol yield on consumed sugars was observed to be 0.38 (±0) g ethanol produced/ g consumed sugars, maximum theoretical yield. As similar to spruce fermentation, lower amounts of glycerol were present at 96 hours, 2.3g/L and dry weight concentration was calculated to be 3.6 g/L.

5.2 Pulse Experiments

Anaerobic pulse experiments were performed to analyse the stress response and fermentation performance of the strain, VTT C-10883 towards inhibitors. The performance was analyzed by growing the strain under two different conditions: with and without addition of inhibitors to a defined media. Three different inhibitor concentrations were chosen for the pulse experiments, [Low concentration: 1.3 g/L HMF; 0.4 g/L Furfural], [Medium concentration: 2.6 g/L HMF; 0.8 g/L Furfural] and [High concentration: 3.9 g/L HMF; 1.2 g/L Furfural]. The relation between HMF and furfural concentration was selected based on Koppram et al, 2012. The low inhibitor concentration of HMF and furfural was selected based on the results obtained from chemostat experiments (Ask et al, manuscript submitted).

These pulse experiments were performed both in shake flasks and batch bioreactors. Initially, in shake flasks, under anaerobic conditions, three different inhibitor concentrations were pulsed in a minimal volume (50 mL) of defined media with low sugar concentrations (20 g/L glucose and

xylose) and the inhibitor concentration that shown maximum effect on specific growth rate, sugar consumption, xylitol yield and ethanol production rate was chosen to further analyse in batch bioreactors under controlled environmental conditions (pH and temperature). Also, higher sugar concentrations (40 g/L glucose and 50 g/L xylose) were used to obtain higher cell concentration and to take samples for the analysis of intracellular, extracellular metabolites and to measure dry weights.

5.2.1 Anaerobic cultivations in shake flasks

Microbial growth curve typically includes four different phases of growth: Lag phase, log or exponential phase, stationary phase and death phase. Lag phase is a period of time where cells usually get adapted to the surrounding environment (nutrients). Time might vary depending upon the nature of inoculation culture. In exponential or log phase, each cell begins to replicate and remain in their healthiest state. Studies related to enzymes and other cell components are usually performed in this "mid-exponential" phase. In stationary phase, increase or decrease in cell number was not observed due to less nutrient availability in the media. Later, it is followed by death phase where cells actually undergo lysis (Madigan et al, 2006). (Fig: 11)

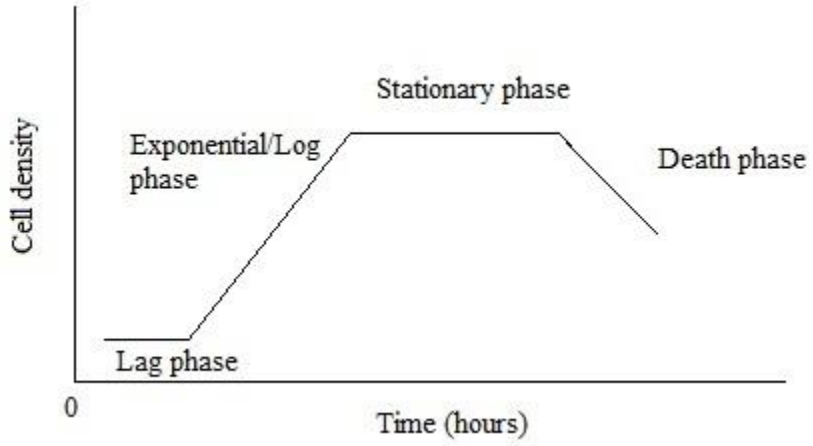


Fig: 11 Microbial Growth Curve

So in order to study the effect of inhibitor on cells, exponential growth phase of the strain was considered as the time for pulsing. Here, VTT C-10883 strain was grown in 12 shake flasks with defined media under anaerobic conditions, considered that each set of 6 shake flask cultures were used to perform different pulse experiments: pulse during exponential growth phase (glucose) and pulse during xylose phase. Three different inhibitor concentrations chosen for these pulse experiments, [Low concentration: 1.3 g/L HMF; 0.4 g/L Furfural], [Medium concentration: 2.6 g/L HMF; 0.8 g/L Furfural] and [High concentration: 3.9 g/L HMF; 1.2 g/L Furfural] were pulsed during the exponential growth phase (glucose) and xylose phase of the VTT C-10883 strain.

5.2.1.1 Pulse during exponential growth phase (glucose)

Pulse experiments during exponential growth phase (glucose) were carried out in 6 anaerobic shake flask cultures and analysed for 78 hours. Among 6 shake flask cultures, one was used as a control and the remaining 5 shake flask cultures were pulsed with three different inhibitor concentrations (medium and high inhibitor concentration were analysed in duplicates) when the OD was around 1.From each shake flasks, a sample volume of about 2ml was taken at 0 hour, each hour (continued for first five hours) after the cultures were pulsed during exponential phase and at varied time intervals during stationary phase for OD measurement and HPLC analysis.

Fig: 12 represents quantitative data: sugar consumption (glucose and xylose), ethanol production, cell concentration (OD_{600}), HMF and furfural consumption obtained from sample with no inhibitor pulse **(a)** and samples pulsed with three different inhibitor concentrations **(b, c and d)**.

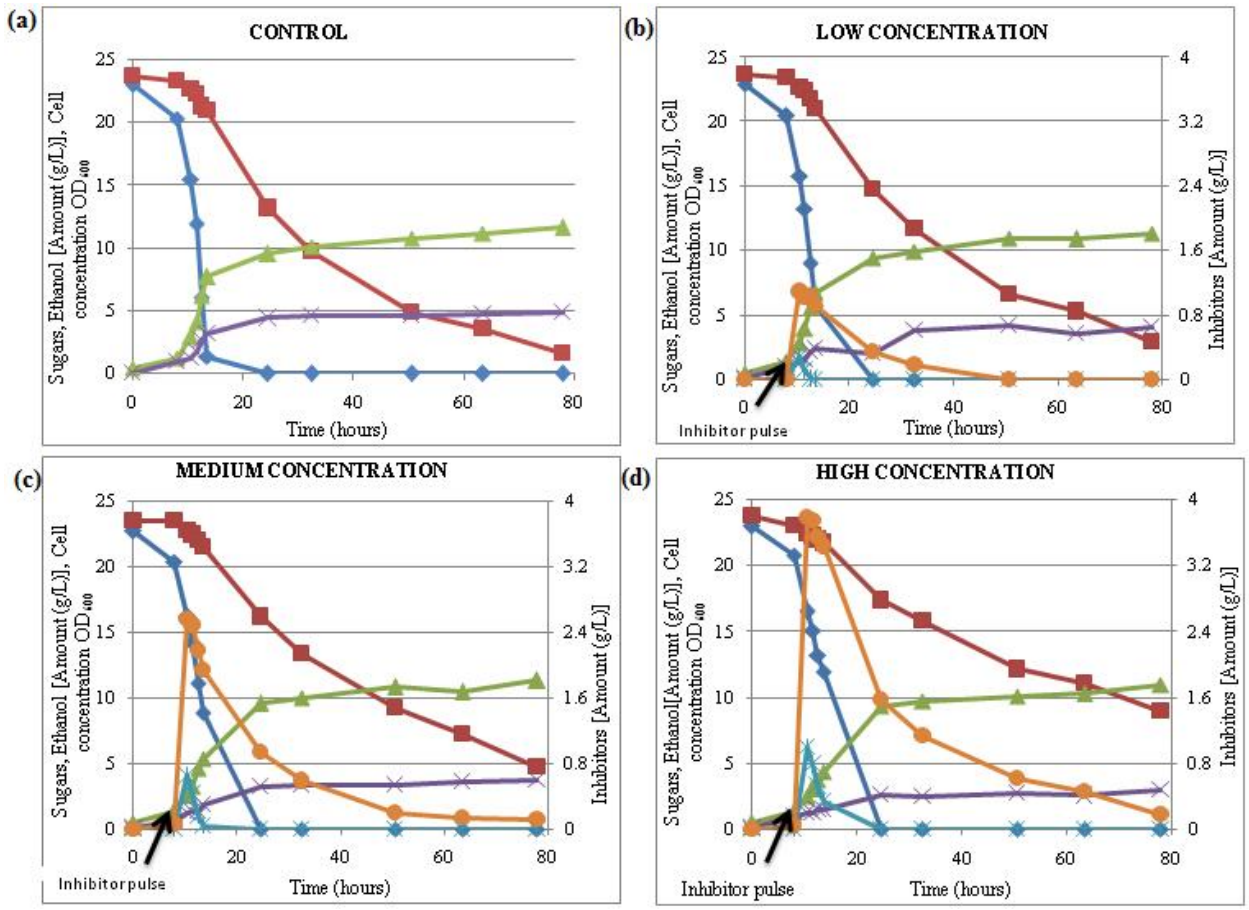


Fig: 12 Pulse during exponential growth phase (glucose) Representation of quantitative data obtained from sample with no inhibitor pulse (a) and samples pulsed with three different inhibitor concentrations during glucose phase (b, c and d). Sugars, ethanol and cell concentration (OD_{600}) have been plotted in primary vertical axis; inhibitors in secondary vertical axis and time in primary horizontal axis.

*The quantitative data points represented in figure c and d were obtained from the average data points of duplicates. In figure (b, c and d), a black arrow represents cell concentration (OD_{600}) at which the inhibitors were pulsed.

In control sample, the cells consumed glucose approximately for 20-22 hours and during this phase, a steady increase in cell concentration was observed from OD 0.1 to 4.44. After complete glucose uptake, the cells consumed xylose at a slower rate compared to glucose. The xylose consumption phase lasted approximately for 56-60 hours and at the end of fermentation 1.6 g/L xylose was left. A rise in cell concentration (OD_{600}) was not observed during this xylose phase and at the end of fermentation, final cell concentration (OD) was 4.92.

The addition of inhibitors to the remaining 5 shake flask cultures caused a decrease in cell concentration and final cell concentrations at 78 hours were calculated to be OD 4, 3.71 and 2.97 for low, medium and high concentration inhibitor pulse, respectively. The specific growth rate (μ) of cells in the absence of inhibitor were 0.23 h^{-1}. It gradually decreased with a rise in inhibitor concentration and was calculated to be 0.21 h^{-1}, 0.11 $h^{-1}$and 0.06 h^{-1} for low, medium and high concentration inhibitor pulse, respectively. The data points used to plot the specific growth curve were obtained from the average data points of duplicates for samples pulsed with medium and high inhibitor concentrations.

Higher amounts of ethanol were produced during glucose consumption phase due to complete glucose utilization. A similar average ethanol yield and ethanol concentration was observed in all pulsed samples and control. In control sample, the average xylitol yield and average glycerol yield at the end of batch were 0.36 g xylitol produced / g consumed xylose and 0.05 g glycerol produced / g consumed sugars, respectively. A decrease in average xylitol yield and increase in average glycerol yield were observed mainly in high concentration inhibitor pulsed samples compared to other samples including control. From the quantitative data obtained, average yields of ethanol, xylitol and glycerol; specific conversion rates of HMF and furfural were calculated.

Table: 6 Representation of average yields and specific conversion rates for each inhibitor concentration obtained from quantitative data of pulse experiments during exponential growth phase (glucose).

Pulse during exponential growth phase (glucose)	**Average Yields and Specific conversion rates**				
	Ethanol (g ethanol produced / g consumed sugars)	Glycerol (g glycerol produced / g consumed sugars)	Xylitol (g xylitol produced / g consumed xylose)	HMF conversion rate (C_i-C_f) / [Avg (OD_i, OD_f) *Δt]	Furfural conversion rate (C_i-C_f) / [Avg (OD_i, OD_f) *Δt]
Control	0.25	0.05	0.36	0	0
Low concentration inhibitor pulse	0.25	0.09	0.42	0.02	0.01
Medium concentration inhibitor pulse	0.26(±0.00)	0.09(±0.00)	0.39(±0.01)	0.01	0.02
High concentration inhibitor pulse	0.28(±0.01)	0.18(±0.04)	0.33(±0.00)	0.02	0.01

Note: ± means standard deviation. C_i- initial concentration; C_f - final concentration, OD_i - initial cell concentration, OD_f - final cell concentration, Δt- time difference, Avg- Average.

5.2.1.2 Pulse during xylose phase

The 6 shake flask cultures prepared for pulse experiments during xylose phase were kept in incubator until it reaches xylose phase. As similar to pulse during glucose phase, one shake flask culture was used as a control, one was pulsed with low inhibitor concentration and 2 shake flask cultures were pulsed with medium and high inhibitor concentration, respectively.

These cultures were pulsed 4 hours after completion of glucose i.e., around 28 hours when the OD was around 4 and these strains were analyzed for 144 hours. A sample volume of about 2 ml was withdrawn at 0 hour, every hour (continued for first five hours) after the cultures were pulsed during xylose phase and at varying time intervals after five hours of pulsing for OD measurement and HPLC analysis.

The cell concentration (OD_{600}) decreased only for half an hour after pulsing and at 144 hours it was 4.82 (control) and 5.84, 4.89, 4.71 for low, medium and high inhibitor concentration pulse, respectively. In xylose consumption phase, the specific growth rate of cells in the presence and absence of inhibitor were calculated to be 0.

The three different inhibitor pulsed samples showed similar average ethanol yields and difference in average xylitol yield was noted between control and inhibitor pulsed samples. The average xylitol yield for control sample was 0.41 g xylitol produced / g consumed xylose at 144 hours. Comparatively, less average xylitol yield was observed in inhibitor pulsed samples and calculated to be 0.28, 0.33(± 0.007), 0.31(± 0.013) g xylitol produced / g consumed xylose for

low, medium and high concentration inhibitor pulse, respectively at 144 hours. Lower amounts of pyruvate and acetate were observed in the samples. Glycerol yield increased with a rise in inhibitor concentration compared to control and the yields were 0.07, 0.11 (± 0.003) and 0.15 (± 0.02) g glycerol produced / g consumed sugars.

Fig: 13 shows the quantitative data that represents xylose consumption, ethanol production, cell concentration (OD_{600}), HMF and furfural consumption obtained from no inhibitor pulse sample and samples pulsed with three different inhibitor concentration. From the quantitative data obtained, average yields of ethanol, xylitol and glycerol; average HMF and furfural conversion rates were calculated.

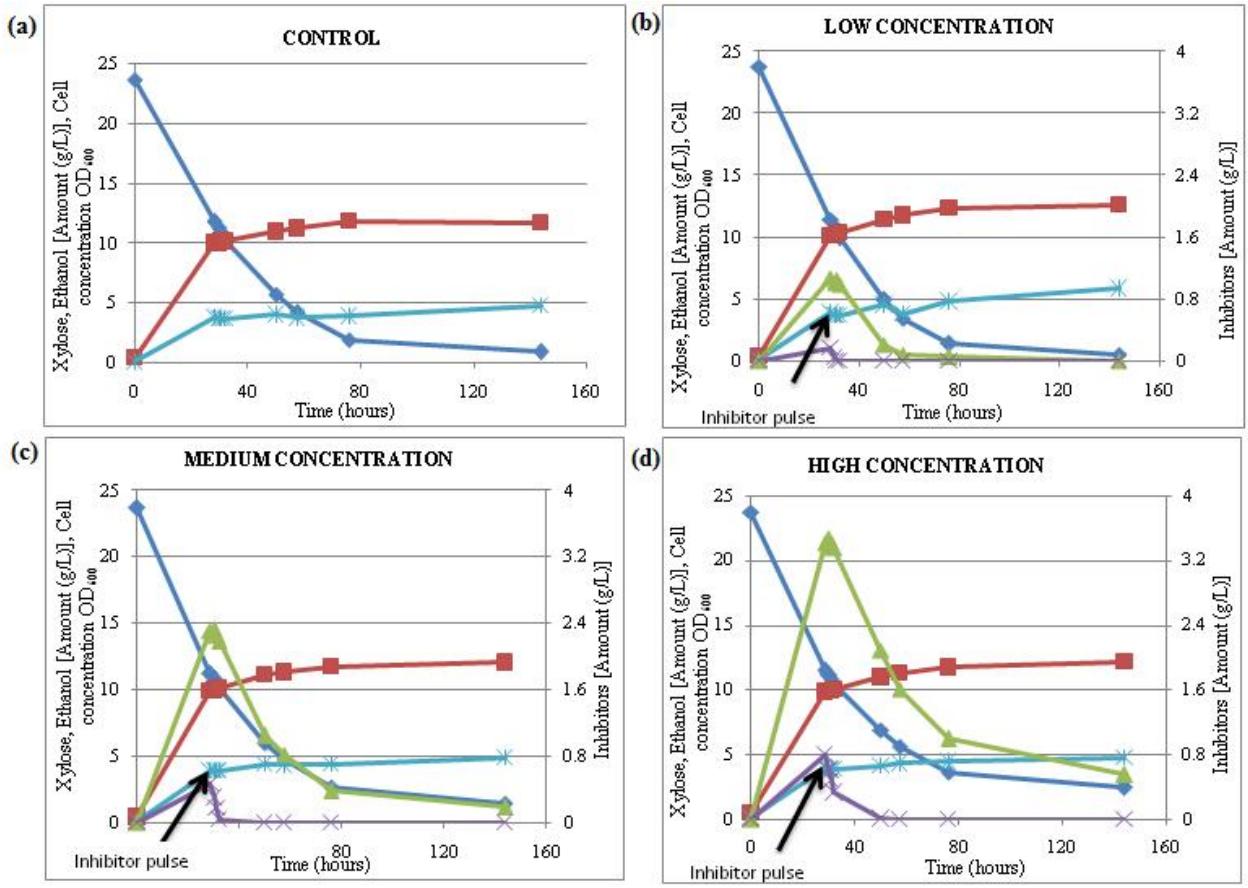


Fig: 13 Pulse during xylose phase Representation of quantitative data obtained from sample with no inhibitor pulse (a) and samples pulsed with three different inhibitor concentrations during xylose phase (b, c and d). Xylose, ethanol and cell concentration have been plotted on primary vertical axis, inhibitors on secondary vertical axis and time on primary horizontal axis.

*The quantitative data points represented in figure b, c and d were obtained from average data points of duplicate measurements. In figure 4(b, c and d), a black arrow pointing represents cell concentration (OD) at which the inhibitors were pulsed.

Table: 7 Representation of average yields and specific conversion rates for each inhibitor concentration obtained from quantitative data of pulse experiments during xylose phase.

Pulse during xylose phase	**Average Yields and Specific Conversion Rates**				
	Ethanol (g ethanol produced / g consumed sugars)	Glycerol (g glycerol produced / g consumed sugars)	Xylitol (g xylitol produced / g consumed xylose)	HMF conversion rate (C_i-C_f) / [Avg (OD_i, OD_f) *Δt]	Furfural conversion rate (C_i-C_f) / [Avg (OD_i, OD_f) *Δt]
Control	0.25	0.05	0.41	0	0
Low concentration inhibitor pulse	0.26	0.07	0.28	0.005	0.025
Medium concentration inhibitor pulse	0.26(±0.00)	0.11(±0.00)	0.33(±0.00)	0.004	0.033
High concentration inhibitor pulse	0.26(±0.00)	0.15(±0.02)	0.31(±0.01)	0.005	0.009

Note: ± means standard deviation. C_i- initial concentration; C_f - final concentration, OD_i - initial cell concentration, OD_f- final cell concentration, Δt- time difference, Avg- average.

5.2.2 Anaerobic batch bioreactor cultivations

The addition of high inhibitor concentration in anaerobic shake flask cultivations showed significant differences in xylitol yield, glycerol yield and specific growth rate compared to the control and the other inhibitor concentrations. So pulse experiments using high concentration inhibitor were carried out to analyse the stress response of the strain, VTT C-10883 in batch bioreactors in scaled up conditions. In bioreactors, anaerobic cultivations were carried out with higher sugar concentrations: 40g/L glucose and 50 g/L xylose in defined medium. The use of higher sugar concentrations was to obtain higher cell mass and to take more samples for the analysis of extracellular metabolites. The four different experiments carried out in bioreactors include glucose and xylose control (addition of no inhibitors), high concentration inhibitor pulse during exponential growth phase (glucose) and high concentration inhibitor pulse during xylose phase.

In glucose and xylose control experiments, no inhibitors were pulsed and each experiment was performed in triplicates. In both the control experiments, a sample volume of 15 ml was withdrawn at regular intervals for OD measurement, HPLC analysis and dry weight measurements. Dry weight measurements were carried out in duplicates. Intracellular metabolite samples were taken when the OD was around 2 and at four hours after completed glucose consumption for glucose control and xylose control experiment, respectively. The specific growth rate for no inhibitor pulse experiments was calculated by taking average of the individual

specific growth rate of the triplicates. In glucose control experiment and xylose control experiment, it was calculated to be 0.28 (± 0.02) h^{-1} and 0 h^{-1}, respectively.

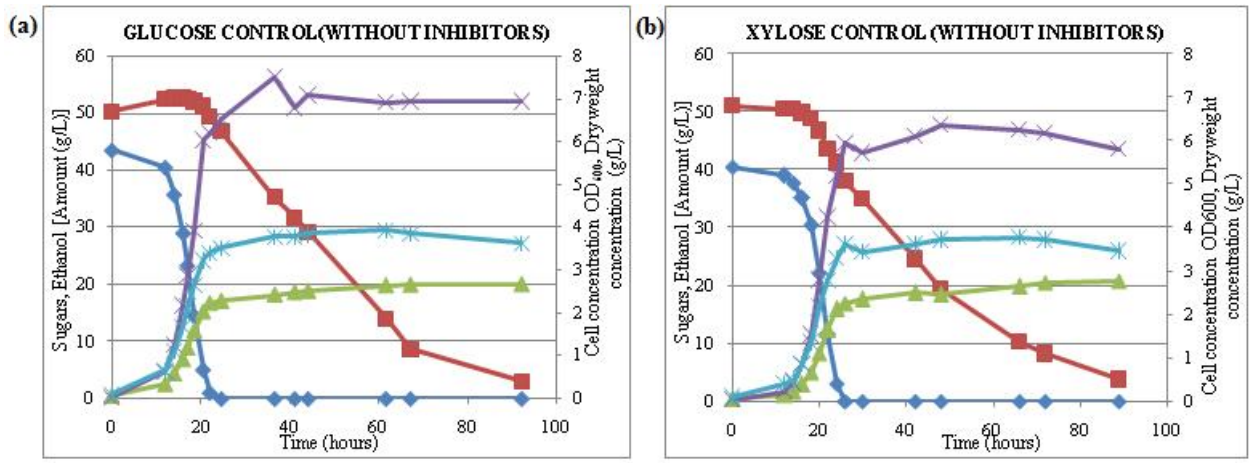


Fig: 14 No inhibitor pulse during glucose and xylose phase Representation of quantitative data obtained from samples with no inhibitor pulse during glucose phase (a) and xylose phase (b). Sugars, ethanol are plotted on primary vertical axis; cell concentration and dry weight concentration have been represented on secondary vertical axis and time on primary horizontal axis.

∗The quantitative data represented in both figure (a) and (b) are obtained from the average data points of the triplicates.

Fig: 14 shows the quantitative data that represents sugar consumption, cell concentration (OD_{600}) and dry weight concentration obtained from samples with no inhibitor pulse during glucose phase **(a)** and xylose phase **(b)**. The average ethanol yield and xylitol yield at the end of batch was calculated to be 0.22 (±0.005) g ethanol produced/ g consumed sugars and 0.44 (±0.009) g xylitol produced/ g consumed xylose, respectively for glucose control experiments. In xylose control experiments, the average ethanol yield and xylitol yield at the end of batch was calculated to be 0.23 (±0.005) g ethanol produced / g consumed sugars and 0.46(±0.005) g xylitol produced/ g consumed xylose, respectively. The energy charge E_c were calculated to be 0.89 (±0.01) and 0.80 (±0.002) for glucose control and xylose control, respectively, at different time points.

The high concentration inhibitor was pulsed when the OD was around 2 and after four hours of complete glucose consumption for glucose pulse and xylose pulse experiments, respectively. A sample volume of 15 ml was withdrawn at different time intervals: 0 hour, each hour after pulsing during exponential growth phase and xylose phase (continued till 6 hours after pulsing) and at varied intervals after 6 hours of pulsing for OD measurement, HPLC analysis and dry weight measurements. Dry weight measurements were carried out in duplicates. In glucose pulse experiment, samples for intracellular metabolites were taken one hour after pulsing when the OD

was around 2 and in xylose pulse, these samples were taken one hour after pulsing i.e., after four hours of complete glucose uptake.

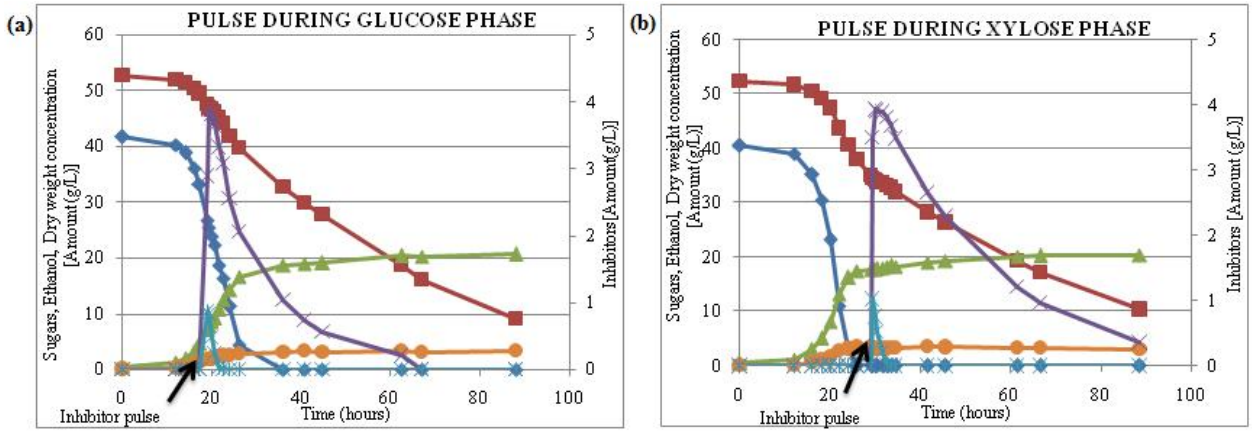


Fig: 15 High concentration inhibitor pulse during glucose and xylose phase Representation of quantitative data obtained from samples with inhibitor pulse during glucose phase (a) and xylose phase (b). Sugars, ethanol and dry weight concentration (in g/L) are plotted on primary vertical axis; inhibitors (in g/L) have been represented on secondary vertical axis and time (in hours) on primary horizontal axis.

∗The quantitative data represented in both figure (a) and (b) are obtained from the average data points of triplicates. In figure (a) and (b), a black arrow pointing represents cell concentration (OD_{600}) at which the inhibitors were pulsed.

Cultures pulsed with high concentration inhibitor during exponential phase shown lower specific growth rate 0.07 (±0.006) h^{-1} than the control. As similar to xylose control cultures, the cultures pulsed with high concentration inhibitor during xylose phase also showed no growth. In pulse during exponential growth phase (glucose), the average ethanol yield was calculated as 0.23 (±0.004) g ethanol produced/ g consumed sugars and in xylose pulse experiment, similar average ethanol yield was observed, 0.24 (±0.004) g ethanol produced/g consumed sugars. In pulse experiments, a decrease in average xylitol yield was observed: 0.41 (±0.003) and 0.38 (±0.003) g xylitol / g consumed xylose for glucose and xylose pulse experiment, respectively compared to control, 0.46(±0.005) g xylitol produced/ g consumed xylose. Also, the average acetate yield was observed to be higher (approximately, 0.04 g acetate produced at the end of fermentation/ g consumed sugars) with high concentration inhibitor pulse during glucose and xylose phases. Energy charges were calculated to be 0.94 ± (0.003) and 0.85 ± (0.02) for glucose pulse and xylose pulse, respectively.

Fig: 15 shows the quantitative data that represents sugar consumption, ethanol production, cell concentration, dry weight concentration, HMF and furfural concentration obtained from samples pulsed with high inhibitor concentration during glucose phase **(a)** and xylose phase **(b)**. From the quantitative data obtained, the average yields of ethanol, xylitol and glycerol; specific conversion rates of HMF and furfural were calculated.

Table: 8 Representation of quantitative data obtained from anaerobic cultivation pulsed with high concentration inhibitor during exponential phase and xylose phase in bioreactors.

Different pulse experiments in anaerobic batch bioreactors	**Average Yields and Specific Conversion Rates**					
	Ethanol (g ethanol produced / g consumed sugars)	Glycerol (g glycerol produced / g consumed sugars)	Xylitol (g xylitol produced / g consumed xylose)	Biomass (g/L dry weight concentration / g consumed sugars)	HMF conversion rate (C_i-C_f) / [Avg (OD_i, OD_f) *Δt]	Furfural conversion rate (C_i-C_f) / [Avg (OD_i, OD_f)*Δt]
Glucose Control	0.22(±0.005)	0.08(±0.009)	0.44(±0.009)	0.04(±0.005)	0	0
Pulse during exponential phase(glucose)	0.23(±0.004)	0.08(±0.002)	0.41(±0.003)	0.04±0.002	0.03	0.09
Xylose control	0.23(±0.005)	0.08(±0.002)	0.46(±0.005)	0.04±0.0006	0	0
Pulse during xylose phase	0.24(±0.004)	0.1(±0.0)	0.38(±0.003)	0.04±0.02	0.01	0.06

Note: ± means standard deviation. C_i- initial inhibitor concentration; C_f - final inhibitor concentration, OD_i - initial cell concentration, OD_f- final cell concentration, Δt- time difference, Avg- average.

6 DISCUSSION

Bioethanol production from lignocellulosic substrates requires a fermenting microorganism that can consume different monosaccharide sugars present in the lignocellulose material even in the presence of inhibitors generated during pretreatment and hydrolysis process. In the first part of study, the industrial strain, GS1.11-26 used to perform SHF experiments with spruce and *Arundo donax* showed better fermentative results than VTT C-10883. The industrial strain carrying XI gene consumed both glucose and inhibitors present in spruce and some amounts of xylose present in *A. donax* were left unconsumed at the end of fermentation. Using this strain, an increase in average ethanol yield on consumed sugars was observed in both spruce (0.42 g/g) and *Arundo* (0.38 g/g).

In the second part of the study, the effect of inhibitors on cell growth and product formation was examined under anaerobic conditions using laboratory strain, VTT C-10883. In pulse experiments (Glucose pulse and xylose pulse), cell growth was initiated with no inhibitor addition. After the addition of high concentration inhibitor (3.9 g/L HMF; 1.2 g/L Furfural) during the exponential growth phase (glucose) and xylose phase showed decrease in cell concentration. Cell growth decreased for first half an hour followed by a recovery of active cell growth. But still the final dry weight and cell concentration (OD_{600}) was observed to be lower compared to the control experiment without inhibitors. In contrast, significant lag phase (4-8h) was observed in the experiments performed by (Liu et al, 2004) where he grew the strains in the synthetic media amended with either HMF or furfural at 3.78 g/L and 1.26 g/L, respectively.

The specific growth rate, μ was very low (0.07 (±0.006) h^{-1}) for the addition of high concentration inhibitor (3.9 g/L HMF and 1.2 g/L furfural) during glucose phase. The cells did not grow on xylose and it has been suggested to be due to less ATP formation during anaerobic xylose metabolism (Rizzi et al, 1989b). It has been observed that the cells were able to convert both HMF and furfural, simultaneously, but the conversion rate of each inhibitor was different. HMF took longer time to convert and it differs for each pulse experiment i.e., in pulse during xylose experiment, the conversion time (59.2 hours) was longer compared to glucose pulse (43 hours). On the other hand, cells consumed furfural at a higher rate (less than 4 hours) which might be due to the available carbon energy.

The cells even in the presence of inhibitors demonstrated active metabolism to consume sugars and produce ethanol. The difference in time was observed in glucose uptake rate in both glucose control (22 hours) and glucose pulse (26-28 hours) experiments. However, in both these experiments glucose was completely consumed and some amounts of xylose were still left at the end of batch. In pulse experiments performed by (Taherzadeh et al, 1999), the addition of 4 g/L furfural to the exponentially growing cultures resulted in lower specific ethanol production rate (0.5 (±0.2 $g.g^{-1}.h^{-1}$) compared to control (1. 6 (±0.1) $g.g^{-1}.h^{-1}$). Similarly, in this study decrease in ethanol production was observed after pulsing, however, at the end of fermentation,

approximately 20 g/L ethanol was present in both pulse and control experiments. A similar average ethanol yield on consumed sugars was obtained in both pulse and control experiments. The energy charge (E_c) was observed to be higher in the samples pulsed during glucose (0.94) and xylose phase (0.85) compared to the samples with no inhibitor pulse. This might be due to less availability of ATP for maintenance.

As mentioned earlier, during anaerobic fermentation of xylose, xylitol is a major by product formed (Kötter and Ciriacy, 1993) due to redox imbalance. NADH can be re-oxidized under anaerobic conditions with the addition of electron acceptors such as furfural (Wahlbom and Hahn-Hägerdal, 2002) and acetoin (Bruinenberg et al, 1983a). In this present study, pulse addition during xylose phase showed significant decrease in xylitol production. Since the conversion of furfural to furfuryl alcohol is NADH dependent (Palmqvist et al, 1999) and regenerated NAD^+ was used for xylitol conversion to xylulose. The resulting xylulose through pentose phosphate pathway (PPP) was then converted to ethanol (Gunsalus et al, 1955). Increase in glycerol production was also observed in pulse during xylose phase which might be due to stress response caused by HMF (Liu et al, 2004).

The average acetate yield on consumed sugars was observed to be higher [0.04 ± (0.00) $g.g^{-1}.h^{-1}$] in the pulse experiments compared to control [0.01 ± (0.0) $g.g^{-1}.h^{-1}$]. This indicates that the addition of furfural causes increase of the NAD^+ pool which resulted in increase of intracellular acetaldehyde accumulation. Thereby, toxic effects of acetaldehyde are avoided because of its conversion of to acetate; generating NAD(P)H. Acetaldehyde concentration higher than 0.3 g/L (Stanley et al, 1993) is toxic to yeast.

7 CONCLUSION

In SHF experiments, the industrial strain, GS1.11-26 showed better fermentative performance; a higher average ethanol yield was observed using this strain in both spruce (0.42 g/g) and *Arundo* (0.38 g/g) fermentation compared to VTT C-10883 strain.

In pulse experiments, VTT C-10883 strain showed tolerance towards inhibitors and efficiently converted both HMF and furfural. The inhibitor addition resulted in no impact of final average ethanol yield. Instead, a decrease in specific growth rate (0.07 (±0.006) h^{-1}) was observed when high concentration inhibitor was pulsed during glucose phase. No growth was observed when high concentration inhibitor was pulsed during xylose phase. The results from pulse experiments show that VTT C-10883 strain has the potential to be improved for further development of tolerant strains towards inhibitors.

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10 APPENDIX

A. Stock solutions for Defined mineral medium preparation

Stock solution for sugars (500 g/L)

550 g of glucose monohydrate was weighed and dissolved in 400 ml of milliQ water and later the volume was made up to 1000 ml. 500 g of xylose was also weighed and dissolved in 400 ml of milliQ water and later the volume was made up to 1000 ml. These two sugar solutions were autoclaved and allowed to cool down to room temperature and stored at 4°C.

Salts-stock solution I (30 X)

Salts	Amount (g/L)
$(NH_4)_2SO_4$	150
KH_2PO_4	70.2
$MgSO_4.7H_2O$	15

The three salt components were dissolved in 750 ml of milliQ water and later the volume was made up to 1000 ml. The salt solution was stored at room temperature.

Salts- stock solution II (20 X)

Salts	Amount (g/L)
$(NH_4)_2SO_4$	100
KH_2PO_4	60
$MgSO_4.7H_2O$	10

The three salt components were dissolved in 750 ml of milliQ water and later the volume was made up to 1000 ml. The salt solution was stored at room temperature.

Salts- stock solution III (2X)

Salts	Amount (g/L)
$(NH_4)_2SO_4$	40
KH_2PO_4	20
$MgSO_4.7H_2O$	4

The three salt components were dissolved in 750 ml of milliQ water and later the volume was adjusted to 1 litre and stored at room temperature.

Phthalate buffer- Stock solution I (5X)

Name	Amount (mmol)
Phthalate	250

51.1 g of sodium phthalate was dissolved in 750 ml of milliQ water and later the volume was made up to 1000 ml. The prepared buffer solution was sterile filtered and stored at room temperature.

Phthalate buffer-Stock solution II (10X)

Name	Amount (mmol)
Phthalate	500

102.2 g of sodium phthalate was dissolved in 750 ml of milliQ water and later the volume was made up to 1000 ml. The prepared buffer solution was sterile filtered and stored at room temperature

Stock solution for vitamins (1000X)

Vitamins	Amount (g/L)
d-Biotin	0.05
Calcium (D+) pantothenate	1.0
Nicotinic acid	1.0
myo-Inositol	25
Thiamine HCl	1.0
Pyridoxine HCl	1.0
Para-amino benzoic acid	0.2

i. Biotin was dissolved in 10 ml of 0.1M NaOH. This solution was then added to 750 ml of milliQ water and pH was adjusted to 6.5 using 1 M HCl.

ii. The rest of the components were dissolved one after the other by maintaining pH at 6.5 and when all the components were dissolved completely, the volume was adjusted to 1 litre with milliQ water.

iii. The prepared vitamin solution was filter sterilized and stored at 4°C.

Stock solution for trace metal elements (1000X)

Trace elements	Amount (g/L)
EDTA	15
$ZnSO_4.7H_2O$	4.5
$MnCl_2.4H_2O$	0.8
$CoCl_2.6H_2O$	0.3
$CuSO_4.5H_2O$	0.3
$Na_2MoO_4.2H_2O$	0.4
$CaCl_2.2H_2O$	4.5
$FeSO_4.7H_2O$	3.0
H_3BO_3	1.0
KI	0.1

EDTA and $ZnSO_4.7H_2O$ were dissolved in 750 ml of milliQ water and pH was adjusted to 6.0 using 1M NaOH.

i. The rest of the trace metal components were dissolved one after the other by maintaining the pH at 6.0. Later when all the components were dissolved, pH was set to 4.0 using 1 M HCl and then the volume was adjusted to 1 liter with miliQ water.

ii. The prepared solution was then filter sterilized, covered with aluminum foil and stored at 4°C.

B. Preparation of Defined mineral medium for Separate Hydrolysis and Fermentation (SHF) process in anaerobic shake flasks

Defined mineral medium for inoculum cultivation

Media components	Volume of stock solution
Glucose (50 g/L)	100 mL
Trace metal solution (2 X)	2 mL
Vitamin solution (2 X)	2 mL
Phthalate buffer (1 X)	100 mL
$(NH_4)_2SO_4$	10 g
KH_2PO_4	6 g
$MgSO_4.7H_2O$	1 g
milliQ water	796 mL

10 g $(NH_4)_2SO_4$, 6 g KH_2PO_4, 1 g $MgSO_4.7H_2O$ and 100 mL of phthalate buffer- stock solution II (10X) were added to 796 mL of milliQ water and mixed well. The rest of the sterile components (glucose, trace metals and vitamins) were added to the salt solution inside the laminar hood and mixed well. The pH of the media was then set to 5 using 1M NaOH. The prepared solution was then sterile filtered and stored at 4°C.

C. Preparation of Defined mineral medium for Separate Hydrolysis and Fermentation (SHF) process in batch bioreactors

Defined mineral medium for inoculum cultivation

Media components	Volume (ml)
Glucose (50 g/L)	40
Vitamin solution (1 X)	1
Trace metal solution (1 X)	1
Salt solution (1 X)	50
Phthalate buffer (1 X)	200
milliQ water	708

50 mL of 20 X salt solution and 200 mL of phthalate buffer stock solution I (5X) were added to 708 mL of milliQ water and mixed well. The rest of the sterile components (glucose, vitamins and trace elements) were added to the prepared solution and mixed well. The pH of the solution was set to 5 using 1M NaOH. The prepared medium was then sterile filtered and stored at 4°C.

Defined mineral medium for propagation of cells in SHF using Spruce

Media	Amount (ml)
Glucose (40 g/L)	56
Trace metal solution (2.7 X)	1.89
Vitamin solution (2.7 X)	1.89
Salt solution (1X)	350
Antifoam	0.2
Inoculum	30
milliQ water	260.02

350 ml of salts- stock solution III (2X) and 0.2 ml of antifoam were added to 288.02 ml of milliQ water. The solution was mixed well and added to the fermenter before autoclavation. The fermenter was then sterilized and allowed to cool down to room temperature. pH, temperature and DO probes were connected to the fermenter. The sterile solutions (glucose, vitamins and trace elements) were added to the fermenter and aerobic batch cultivation for SHF-spruce was initiated by the addition of 30 ml inoculum.

Note: To adjust for evaporation losses during autoclaving, 20 % extra volume of milliQ water, polypropylene glycol and salt solution were added to the fermenter before autoclavation and the extra volume was removed after sterilization using syringe.

Defined mineral medium for propagation of cells in SHF using *Arundo donax*

Media	Amount (ml)
Glucose (20 g/L)	28
Trace metal solution (2.7 X)	1.89
Vitamin solution (2.7 X)	1.89
Salt solution (1X)	350
Antifoam	0.2
Inoculum	30
milliQ water	288.52

350 ml of salts- stock solution III (2X) and 0.2 ml of antifoam were added to 288.02 ml of milliQ water. The solution was mixed well and added to the fermenter before autoclavation. The fermenter was then sterilized and allowed to cool down to room temperature. pH, temperature and DO probes were connected to the fermenter. The sterile solutions (glucose, vitamins and trace elements) were added to the fermenter and aerobic batch cultivation for SHF-*Arundo* was initiated by the addition of 30 ml inoculum.

Note: To adjust for evaporation losses during autoclaving, 20 % extra volume of milliQ water, polypropylene glycol and salt solution were added to the fermenter before autoclavation and the extra volume was removed after sterilization using syringe.

D. Preparation of Defined mineral medium for pulse experiments

Defined mineral medium for anaerobic cultivations in shake flasks

Media components	Volume of stock solution(ml)
Glucose (20 g/L)	40
Xylose (20 g/L)	40
Vitamin solution (1 X)	1
Trace metal solution (1 X)	1
Salt solution (1 X)	50
Phthalate buffer (1 X)	200
milliQ water	668

50 ml of salts- stock solution II (20X) was added to 668 ml of milliQ water and mixed well. The rest of the sterile solutions (glucose, xylose, vitamins, trace metals and phthalate buffer) were added to the (salt + milliQ water) solution inside the laminar hood. The pH of the solution was then set to 5 using 1M NaOH. The prepared solution was filter sterilized and stored at 4°C

Note: To maintain anaerobic growth in shake flasks, final concentration of 10 mg/L Ergosterol and 420 mg/L Tween 80 were added to the Defined mineral medium.

Defined mineral medium for anaerobic batch fermentation in bioreactors

Media components	Volume of stock solution(ml)
Glucose (40 g/L)	120
Xylose (50 g/L)	150
Vitamin solution (2 X)	3
Trace metal solution (2 X)	3
Salt solution (1.5 X)	75
Ergosterol/Tween 80	1.5
Polypropylene glycol (P 200)	0.5
Inoculum	75
milliQ water	1072.5

0.5 ml of polypropylene glycol and 75 ml of salts-stock solution I (30X) were added to 1072.5 ml of milliQ water and mixed well. This prepared solution was added to each fermenter before autoclaving and pH, DO and OD probes were inserted to each fermenter. Later these fermenters were sterilized and allowed to cool down to room temperature. The pH, DO and OD probes were then connected to each fermenter and the solution present inside was sparged with nitrogen gas before the addition of measured volumes of sterile solutions (glucose, xylose, vitamins, trace elements, ergosterol/tween 80). The fermentation was initiated by the addition of 75 ml inoculum with OD_{600}0.025.

Note: To adjust for evaporation losses during autoclaving, 5 % extra volume of milliQ water, polypropylene glycol and salt solution were added to each fermenter before autoclavation and the extra volume was later removed after sterilization using syringe.

E. Enzymatic hydrolysis in SHF process

WIS concentration (initial) of the pretreated lignocellulosic material

i. *Arundo* = 19 %
ii. Spruce = 21.05%

Stock concentration of enzymes

Enzymatic hydrolysis method	Enzyme name	Amount
Enzymatic hydrolysis of *Arundo*	Celluclast	143 mg/mL
	Novozymes	8451 nkat/mL
	Xyl11	49% protein content
Enzymatic hydrolysis of spruce	Cellobiohydrolases CBH I	44.72 mg/mL
	Cellobiohydrolases CBH II	133.44 mg/mL
	Endoglucanases EG II	96.17 mg/mL
	β- Glucosidase (β-G)	113mg/mL

F. Stock concentration of inhibitors

S.No	Experiments		HMF concentration [in Molarity (M)]	Furfural concentration [in Molarity (M)]
1	Pulse experiments in anaerobic shake flasks		5.3	12.1
2	Pulse experiments in anaerobic batch bioreactors	Pulse during glucose phase	7.739	12.1
		Pulse during xylose phase	5.68	12.1

G. Stock concentration of Ergosterol and Tween 80

i. Ergosterol = 10 g/L
ii. Tween 80 = 420 g/L

Preparation of HPLC standards for the analysis of sugars, acids and inhibitors

H. Separate hydrolysis and fermentation (SHF) - *Arundo donax* and spruce

Enzymatic hydrolysis and fermentation in bioreactors

Stock solution for sugars, xylitol, acids, alcohol and inhibitors

50 g/L glucose
50 g/L xylose

50 g/L mannose
20 g/L xylitol
25 g/L cellobiose
25 g/L galactose
25 g/L arabinose
40 g/L ethanol
10 g/L glycerol
10 g/L HMF
10 g/L furfural

Standards for sugars, xylitol and acids

Standard 1 = 500 µl milliQ H_2O (50 g/L)

Standard 2 = 250 µl stock + 250 µl milliQ H_2O (25 g/L)

Standard 3 = 125 µl stock + 375 µl milliQ H_2O (12.5 g/L)

Standard 4 = 50 µl stock + 450 µl milliQ H_2O (5 g/L)

Standard 5 = 10 µl stock + 490 µl milliQ H_2O (1 g/L)

Note: Prepare standards on ice.

Table: 9 Representation of final concentrations (g/L) of all compounds in standards

Compounds	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
Glucose	50	25	12.5	5	1
Xylose	50	25	12.5	5	1
Mannose	50	25	12.5	5	1
HMF	10	5	2.5	1	0.5
Furfural	10	5	2.5	1	0.5
Ethanol	40	20	10	4	0.8
Xylitol	20	10	5	2.5	0.5
Cellobiose	25	12.5	6.25	2.5	0.5
Galactose	25	12.5	6.25	2.5	0.5
Arabinose	25	12.5	6.25	2.5	0.5
Glycerol	10	5	2.5	1	0.2

Note: Samples from enzymatic hydrolysis and fermentation were diluted 5 times and analysed in HPLC.

Fermentation in anaerobic shake flasks

Stock solution for glucose/xylose/xylitol, acids, alcohol and inhibitors

750 mL of glucose/xylose/xylitol stock solution and 750 mL of acid stock solution used to analyse the bioreactor samples from pulse experiments were mixed to obtain these given concentrations

25 g/L glucose
25 g/L xylose
10 g/L xylitol
5 g/L acetate
5 g/L glycerol
15 g/L ethanol
2.5 g/L HMF
2.5 g/L furfural
0.5 g/L pyruvate
0.5 g/L succinate

Standards for glucose/xylose/xylitol, acids, alcohol and inhibitors

Standard 1 = 100 µl stock + 400 µl eluent (20 g/L)

Standard 2 = 80 µl stock + 420 µl eluent (10 g/L)

Standard 3 = 60 µl stock + 440 µl eluent (5 g/L)

Standard 4 = 40 µl stock + 460 µl eluent (1 g/L)

Standard 5 = 20 µl stock + 480 µl eluent (0.1 g/L)

Note: Prepare standards on ice.

Table: 10 Representation of final concentrations (g/L) of all compounds in standards

Compounds	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
Glucose	25	10	5	1	0.1
Xylose	25	10	5	1	0.1
Xylitol	10	14	7	0.2	0.02
HMF	2.5	1	0.5	0.1	0.01
Furfural	2.5	1	0.5	0.1	0.01
Ethanol	15	6	3	0.6	0.06
Acetate	5	2	1	0.2	0.02
Glycerol	5	2	1	0.2	0.02
Pyruvate	0.5	0.2	0.1	0.02	0.002
Succinate	0.5	0.2	0.1	0.02	0.002

Note: Fermentation samples were diluted 5 times and analysed in HPLC.

Pulse experiments in anaerobic shake flasks

Stock solution for glucose/xylose/xylitol

25 g/L glucose
25 g/L xylose
12 g/L xylitol

To prepare 25 ml of glucose/xylose/xylitol solution, 0.625 g of each sugar and 0.3 g of xylitol were added to a 25 ml standard flask and milliQ water was added till the level marked on the flask. 25 ml solution was divided in 16 eppendorf tubes with 1.5 ml in each. The eppendorf tubes were then stored in the freezer.

Stock solution for acids and alcohol

4 g/L acetate
2 g/L glycerol
1 g/L pyruvate
1 g/L succinate
30 g/L ethanol

To prepare 25 ml acid solution, 0.138 g of Na-acetate, 0.031 g of pyruvate, 0.025 g of succinate, 39.6 µl of glycerol and 951 µl of ethanol were added to a 25 ml standard flask and milliQ water was added till the level marked on the flask. 25 ml solution was divided in 16 eppendorf tubes with 1.5 ml in each. The eppendorf tubes were stored in the freezer.

Stock solution for inhibitors

5 g/L HMF
5 g/L furfural

To prepare 25 ml inhibitor solution, 128 µl of5.3 M HMF, 107.8 µl of 12.1 M furfural were added to a 25 ml standard flask and milliQ water was added till the level marked on the flask. 25 ml solution was divided in 16 eppendorf tubes with 1.5 ml in each. The eppendorf tubes were stored in the freezer.

Eluent

5mM H_2SO_4 in milliQ water

Prepare by adding 25 ml of 1M H_2SO_4 to milliQ water. The total volume is made to be 5 litres.

Standards for glucose/xylose/xylitol, acids and inhibitors

Standards for glucose/xylose/xylitol

Standard 1 = 500 µl stock (25 g/L)

Standard 2 = 300 µl stock + 200 µl milliQ H_2O (15 g/L)

Standard 3 = 100 µl stock + 400 µl milliQ H_2O (5 g/L)

Standard 4 = 50 µl stock + 450 µl milliQ H_2O (2.5 g/L)

Standard 5 = 10 µl stock + 490 µl milliQ H_2O (0.5 g/L)

Standards for acids and alcohol

Standard 1 = 500 µl stock (30 g/L)

Standard 2 = 250 µl stock + 250 µl milliQ H_2O (15 g/L)

Standard 3 = 125 µl stock + 375 µl milliQ H_2O (7.5 g/L)

Standard 4 = 41.7 µl stock + 458.3 µl milliQ H_2O (2.5 g/L)

Standard 5 = 16.66 µl stock + 483.3 µl milliQ H_2O (1 g/L)

Standards for inhibitors

Standard 1 = 500 µl stock (5 g/L)

Standard 2 = 250 µl stock + 250 µl milliQ H_2O (2.5 g/L)

Standard 3 = 100 µl stock + 400 µl milliQ H_2O (1 g/L)

Standard 4 = 10 µl stock + 490 µl milliQ H_2O (0.1 g/L)

Standard 5 = 1 µl stock + 499 µl milliQ H_2O (0.001 g/L)

Note: Prepare standards on ice

Table: 11 Final concentrations (g/L) of compounds present in all standards

Compounds	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
Glucose	25	15	5	2.5	0.5
Xylose	25	15	5	2.5	0.5
Xylitol	12	7.2	2.4	1.2	0.24
HMF	5	2.5	1	0.1	0.001
Furfural	5	2.5	1	0.1	0.001
Ethanol	30	15	7.5	2.5	1
Acetate	4	2	1	0.33	0.13
Glycerol	2	1	0.5	0.167	0.067
Pyruvate	1	0.5	0.25	0.083	0.033
Succinate	1	0.5	0.25	0.083	0.033

I. Pulse experiments in anaerobic batch bioreactors

Stock solution for glucose/xylose/xylitol

50 g/L glucose
50 g/L xylose
20 g/L xylitol

To prepare 25 ml of glucose/xylose/xylitol solution 1.25 g of each sugar and 0.5 g of xylitol were added to a 25 ml standard flask and milliQ water was added till the level marked on the flask. 25 ml solution was divided in 16 eppendorf tubes with 1.5 ml in each. The eppendorf tubes were then stored in the freezer.

Stock solution for acids, alcohol and inhibitors

10 g/L acetate
10 g/L glycerol
30 g/L ethanol
5 g/L HMF
5 g/L furfural
1 g/L pyruvate
1 g/L succinate

To prepare 25 ml of acid solution, 0.347 g of Na-acetate, 0.031 g of pyruvate, 0.025 g of succinate, 198 µl of glycerol, 951 µl of absolute ethanol, 128 µl of HMF, 1078 µl furfural were added to a standard 25 ml flask and milliQ water was added till the level marked on the flask. Divide the solution in 16 eppendorf tubes with 1.5 ml in each. The eppendorf tubes were stored in the freezer.

Standards for glucose/xylose/xylitol and acids

Standards for glucose/xylose/xylitol

Standard 1 = 500 µl stock (50 g/L)

Standard 2 = 350 µl stock + 150 µl eluent (35 g/L)

Standard 3 = 200 µl stock + 300 µl eluent (20 g/L)

Standard 4 = 100 µl stock + 400 µl eluent (10 g/L)

Standard 5 = 10 µl stock + 490 µl eluent (1 g/L)

Standards for acids, alcohol and inhibitors

Standard 1 = 500 µl stock (30 g/L)

Standard 2 = 333.3 µl stock + 166.7 µl eluent (20 g/L)

Standard 3 = 166.7 µl stock + 333.3 µl eluent (10 g/L)

Standard 4 = 16.67 µl stock + 483.33 µl eluent (1 g/L)

Standard 5 = 1.67 µl stock + 498.33 µl eluent (0.1 g/L)

Note: Prepare standards on ice.

Table: 12 Representation of final concentrations (g/L) of all compounds in standards

Compounds	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
Glucose	50	35	20	10	1
Xylose	50	35	20	10	1
Xylitol	20	14	8	4	0.4
HMF	5	3.33	1.67	0.167	0.0167
Furfural	5	3.33	1.67	0.167	0.0167
Ethanol	30	20	10	1	0.1
Acetate	10	6.67	3.33	0.33	0.033
Glycerol	10	6.67	3.33	0.33	0.033
Pyruvate	1	0.6	0.33	0.033	0.0033
Succinate	1	0.6	0.33	0.033	0.0033